

HIV-1 MATERNAL TRANSMISSION AND PEDIATRIC DISEASE
OCCUR IN THE ABSENCE OF DETECTABLE INFECTION IN
CD14⁺ MONOCYTES AND IN DIRECT ASSOCIATION WITH
PROVIRAL COPY NUMBER IN CD4⁺ T LYMPHOCYTES

By

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I dedicate this thesis to my dear parents, Zélia and
Maurício Aleixo.

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Pediatric HIV infection is a serious health problem in the world today. Most children are infected through their mothers. However, not all infants born to HIV positive mothers become infected. Furthermore, vertical transmission varies in different geographical regions. Why some mothers transmit the virus and others do not, is not well understood. Multiple factors are associated with mother-to-child transmission, including disease stage of the mother, virus phenotype, levels of maternal virus and obstetrical factors. Hypothesizing that characteristics of the maternal virus are critical for pediatric infection, peripheral blood mononuclear cells (PBMC) from mothers and infants were examined to determine: 1. main target cell of HIV-1 (CD4⁺ T cells or monocytes); 2.

association of cell-associated proviral levels with transmission (mothers treated with zidovudine [ZDV] versus untreated mothers); and 3. association of pediatric proviral levels with timing of infection (in utero versus perinatal) and progression of disease. The conclusions of this study are as follows: 1. Blood monocytes are not a virus reservoir *in vivo*, and CD4⁺ T lymphocytes are the main cell type infected in mothers and infants; 2. Virus with macrophage-tropic characteristics are detected in PBMC cultures; 3. Levels of maternal virus in CD4⁺ T cells can predict vertical transmission in the absence of ZDV; 4. ZDV does not reduce transmission by lowering maternal proviral burden; 5. Levels of infected CD4⁺ T cells in infants are associated directly with acute and chronic stage disease; 6. In infants, memory and naive CD4⁺ T cells are equally infected by HIV-1, while in children >2 years of age, the memory T cell subtype is preferentially infected. These findings will contribute to the understanding of the immunopathogenesis of HIV-1 pediatric infection and maternal-infant transmission, critical to the development of drugs and better therapeutic strategies.

CHAPTER 1 INTRODUCTION

The human immunodeficiency virus (HIV) was first described as the causative agent of AIDS (acquired immune deficiency syndrome) in 1983 by a group leaded by L. Montagnier, at the Pasteur Institute in France (10). Since then, HIV has been intensively studied for several research groups worldwide. It is not known whether HIV is a virus recently transmitted to humans or if it has been present in man for many generations. HIV is spread by different routes including sexual contact, exposure to infected blood products and maternal transmission to the child (40).

By the end of 1992, it was estimated by the World Health Organization (WHO) that 13 million persons, including 1 million children, were infected with HIV in the world. Of all cases in children reported in the United States at that time, 90% were attributable to vertical transmission (210). The astonishing figures in the rapid increase of HIV disease in the world urge the scientific community to develop means of stopping this epidemics. With this in mind, my studies were oriented towards understanding clinical and virological factors in maternal-infant transmission of HIV type 1.

The HIV-1 Virion

Classification. HIV-1 is a lentivirus member of the family of *Retroviridae* (retrovirus), which also includes oncogenic retroviruses and spumaviruses. Retroviruses are characterized by the presence of reverse transcriptase in the virions. Lentiviruses exist in different species and include visna and maedi viruses of the sheep, equine infectious anemia virus (EIAV), caprine arthritis-encephalitis virus, bovine immune deficiency virus, feline immune deficiency virus and simian immune deficiency virus, besides human immune deficiency viruses types 1 and 2 (54) (Fig. 1.1).

This subfamily of retroviruses causes slowly developing disease, characterized by a long incubation period and extended course. A factor in the protracted course of HIV disease could be the high mutability of the HIV-1 genome.

Structure. By electron microscopy, the HIV-1 virion has an icosahedral structure (75) containing 72 spikes in its surface, the envelope glycoproteins (gp) (59, 204). These proteins are cleaved from a common precursor gp160 by a cellular protease, into an external surface protein gp120 and a transmembrane protein gp41 (120), which bind in a noncovalent way (90). The envelope gp120 is comprised of 5 hypervariable regions, V1-V5. A small site within gp120, consisting of 24 amino acids localized in the third variable region (V3), is HIV's principal neutralizing domain (71, 212). The binding site for the cellular receptor CD4 is localized in

Oncoviruses

Rous sarcoma virus (chickens)
Feline leukemia virus (FeLV)
Bovine leukemia virus (BLV)
Human T-leukemia virus, Type I (HTLV-I)
Human T-leukemia virus, Type II (HTLV-II)

Spumaviruses

Simian foamy virus (SFV)
Bovine syncytial virus (BSV)
Feline syncytium-forming virus (FSFV)
Human nasopharyngeal carcinoma virus (NPCV)

Lentiviruses

Equine infectious anemia virus (EIAV)
Caprine arthritis encephalitis virus (CAEV)
Visna virus (sheeps)
Bovine immunodeficiency virus (BIV)
Feline immunodeficiency virus (FIV)
Human immunodeficiency virus (HIV)
Simian immunodeficiency virus (SIV)
Chimpanzee immunodeficiency virus (CIV)

Figure 1.1. RNA viruses. Animal RNA viruses are classified into 3 families: oncoviruses, or transforming viruses, cause cancer; spumaviruses, also called "foamy" viruses because of the appearance they induce in cells they infect; and

gp120, in a region between V4 and V5 (206). The envelope protein gp41 seems to be responsible for the fusion of viral and cellular membranes (112). The core is cone-shaped and the 4 nucleocapsid (NC) proteins are proteolytically cleaved from a 55 kDa precursor by the HIV-1 protease (PR) into p24, p17, p9, and p7 (89). The p24 Gag protein is the main component of the inner NC. Inside the core there are 2 identical copies of single stranded RNA. The viral enzymes reverse transcriptase (RT), integrase (IN) and PR are cleaved from the Pol precursor (189), and together with the NC proteins p9 and p7, they are found closely associated to the RNA. RT acts to form a double-stranded DNA copy of the virus RNA and IN is involved in viral integration. The NC p17 protein associates with the inner surface of the lipid bilayer to stabilize the virion (82). A diagram of the HIV-1 virion is shown in Fig. 1.2.

Genomic organization. Lentiviruses are distinguished from other retroviruses by the presence of a complex genome. Most oncogenic retroviruses that are capable of replication contain only 3 genes (*gag*, *pol*, and *env*) (197). However, HIV-1 contains not only these 3 essential genes but also at least 6 additional genes (*tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*) (82) (Fig. 1.3).

The HIV-1 genome is approximately 9.8 Kb. It has a 5' and a 3' long terminal repeat (LTR), composed of U3, R, and U5 regions, which contain regulatory sequences recognized by various cellular transcription factors. The TATA box homology

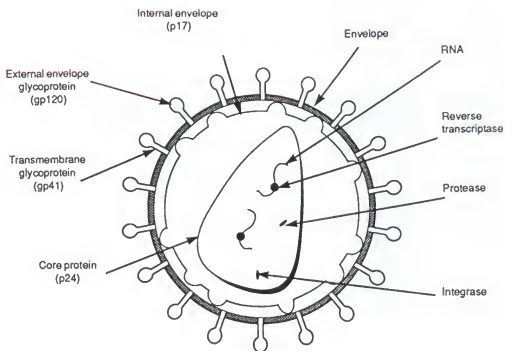


Figure 1.2. Diagram of the HIV-1 virion. Envelope (gp120 and gp41) and nucleocapsid (p17 and p24) proteins are identified. Also shown are viral enzymes reverse transcriptase, protease, and integrase, and the virus diploid RNA genome (Modified from ref. 3).

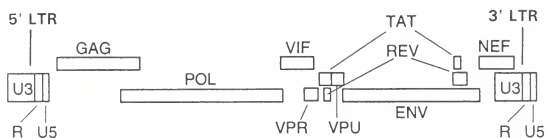


Figure 1.3. Genomic structure of HIV-1. Represented are the 5' and 3' long terminal repeats (LTRs), structural (*gag*, *pol*, *env*), regulatory (*tat*, *rev*) and accessory (*nef*, *vif*, *vpr*, *vpu*) genes (Modified from ref. 133).

is an essential element for *trans*-activation and serves as the binding site for the TATA box DNA-binding protein TFIID, functioning in transcription initiation. Immediately upstream the TATA box are 3 G-C-rich sequences which bind the cellular transcription factor SP1. Within the enhancer element is the consensus recognition sequence for the DNA-binding protein nuclear factor kappa B (NF κ B). Additional DNA-binding proteins are nuclear factor of activated T cells (NF κ A-1), activation protein-1 (AP-1), and the negative regulatory element (NRE). DNA-binding proteins which bind to the leader region include CTF/NF-1 and leader-binding protein (LBP-1). The *trans*-activation response sequence (TAR)-binding protein UBP-1 binds directly to the TATA box homology (187).

Protein products have been identified from 10 open reading frames in the HIV-1 genome. The primary transcript of HIV-1, Gag-Pol precursor p160, is translated into Gag precursor p55 which gives rise to 5 structural proteins, and the Pol precursor protein which is cleaved into the viral replication enzymes. Splicing events producing many subgenomic mRNA are important for the synthesis of other viral proteins. The *rev* gene appears to determine the amount of unspliced to singly and multiply spliced mRNA. The envelope glycoproteins gp120 and gp41 are made from a precursor gp160, a single-spliced message from the full-length viral mRNA.

Gene products of other spliced mRNAs give rise to at least 6 regulatory and accessory proteins, namely *tat* and *rev*

(regulatory) (53, 153), and *nef*, *vif*, *vpr*, and *vpu* (accessory) (16, 37, 73, 79, 194). Major functions of the protein products are: Tat, transactivation; Rev, regulation of viral protein expression; Nef, virus suppression, signal transduction, and cell activation; Vif, increases virus infectivity and cell-to-cell transmission; Vpr, helps in virus replication; and Vpu, helps in virus release.

Heterogeneity of HIV. Two major types of AIDS viruses, HIV-1 and HIV-2, can be identified. HIV-2 differs by more than 55% from HIV-1, the major difference residing in the envelope glycoproteins (85). Antibodies to HIV-2 generally cross-react with Gag and Pol proteins of HIV-1, but envelope proteins may not be detected (76). HIV-2 glycoproteins seem to cross-react with envelope proteins from SIV, and because of the marked similarities in their sequences, it appears that HIV-2 was derived from SIV (115). Individuals infected with HIV-2 survive longer than with HIV-1 infection, suggesting that HIV-2 is less pathogenic to humans (205).

Based on the viral envelope sequences, 9 subtypes of HIV-1 (A to I) have been identified in the world (134) (Fig. 1.4). The clades differ from each other by at least 20% in the amino acid composition of the envelope region, and 15% in the Gag region. Within each clade, the differences in Env can be up to 10%, and up to 8% in the Gag region (133). Clade A is found in Central Africa, B in North and South America and in Europe, subtype C in South Africa and India, subtype D in Central

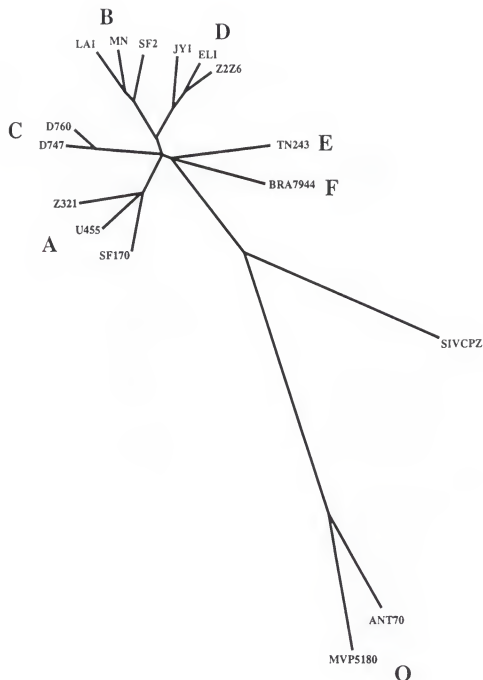


Figure 1.4. A neighbor-joining tree showing the classification of HIV-1 sequences into 6 clusters, A through F, based upon envelope coding sequences. A chimpanzee virus sequence (SIVCPZ) and 2 highly divergent Cameroonian sequences designated O (for "outlying"), are also shown. The sequence subtypes A through F are each 30% different from one another and about 50% different from the O group sequences (Modified from ref. 106).

Africa, subtype E in Thailand, and F in Brazil (107, 129) and Romania (57). G, H, and I are clades recently found in Africa, Russia, and Taiwan (133).

Viruses recovered from one individual have several conserved restriction enzyme sites, which identify the virus as coming from the same person (86). Viruses from the same patient form a heterogenous population, referred to as *quasispecies* (80), and the diversity within an individual usually ranges up to no more than 7% (133). At least 6% of the viral genome can differ among strains from different individuals. Isolates can vary in both synonymous (mutations that do not affect amino acid expression) and nonsynonymous (mutations that affect amino acid expression) sequence changes. Up to 40% nonsynonymous mutations can be observed in regulatory and envelope gene products (132). The viral RT appears to be responsible to changes in the genome, since up to 10 base changes may occur per replicative cycle (147).

Life cycle. HIV-1 infects cells expressing at their surface the CD4 protein, which acts as the viral receptor. The 55 kDa CD4 molecule has 2 important functions in immune responses; it serves as a cell-cell adhesion molecule and it also functions as a signal transducer (1). The major cellular targets for HIV-1 *in vivo* are the CD4⁺ T lymphocyte and tissue macrophages. The HIV-1 envelope protein gp120 binds to CD4, while gp41 causes its fusion to the cell membrane. A diagram of the life cycle of HIV-1 is shown in Fig. 1.5.

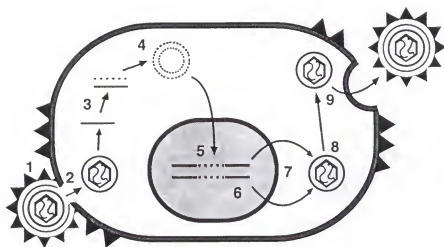


Figure 1.5. HIV-1 life cycle. The steps are as follows: 1, attachment; 2, uncoating; 3, reverse transcription; 4, circularization; 5, integration; 6, transcription; 7, translation; 8, core assembly; 9, final assembly and virus budding. (—, RNA; ----, DNA) (Modified from ref. 106).

After internalization, the viral RNA still associated with the core proteins is reverse transcribed, eventually forming double-stranded DNA. Binding of the tRNA primer starts the process. Synthesis of DNA to the end of the 5' R region (minus-strand strong stop DNA), template switching and elongation to complete the minus strand up to the primer binding site (PBS) follow. Synthesis of the 5' LTR is initiated from the 3' end of the minus-strand DNA (plus-strand strong stop). A second template-switching occurs and synthesis of double-stranded DNA molecule is completed (198). The first-strand DNA copy of the viral RNA is mediated by the virus RT. Second-strand DNA synthesis, also mediated by RT, initiates after partial degradation of the RNA by viral ribonuclease H (82). The DNA copies are transported to the nucleus as a preintegration complex with core and IN proteins, where the viral DNA integrates into the host genome. The integration process is essential for virus replication and it appears to be random (78), although recent reports suggest that HIV-1 may integrate preferentially into L1s (human L1 elements) repetitive elements in the human genome (188).

The HIV-1 LTR is the virus promoter region. Cellular transcription factors seem to be of utmost importance in the initiation of early mRNA transcription and include NF- κ B, AP-1 and SP-1, among others (192).

Following integration, double-spliced transcripts encoding the genes *tat*, *rev*, and *nef*, are the earliest mRNA

species produced (99). In the late stages, structural and enzymatic proteins encoded by *gag-pol* and *env* are produced, and the transition between the synthesis of early-regulatory and late-structural products appears to be dependent on Rev (153).

Assembly of the HIV-1 virion involves aggregation of the core in the cytoplasm, which contains the viral RNA, Gag and Pol proteins. The assembled virion buds through the plasma membrane, when it acquires the lipid bilayer and *env* gene products (128).

Immunopathogenesis of HIV-1 Infection

Natural history. HIV-1 disease causes a variety of symptoms, from apparently silent infection to clinical disease. A main feature of the immunopathogenesis of HIV-1 is the depletion of CD4⁺ T lymphocytes, eventually leading to immune deficiency and AIDS. A diagram of events that happen during HIV-1 infection is shown in Fig. 1.6.

In the initial days following acute infection, high levels of virus replication take place in the activated lymphocytes in the lymph nodes. Up to 5×10^6 virions/ml of plasma can be detected during this stage (145), the extent of virus production most likely reflecting the susceptibility of the individual's PBMC to the virus. During this stage the numbers of CD8⁺ T cells rise, as seen in other viral infections (14). The high viremia is a transitory process and

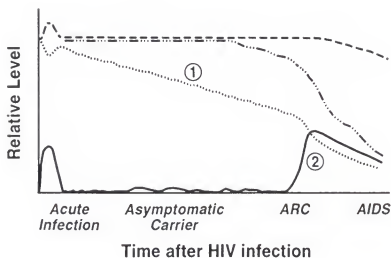


Figure 1.6. Diagram of events occurring after HIV-1 infection. High levels of virus (—) can be detected in the blood during acute phase of infection, before seroconversion. Subsequently this viremia is reduced to lower levels (phase 1). With the onset of clinical symptoms-AIDS, high levels of viremia are detected once again (phase 2). The $CD4^+$ T cell number (...) decreases during acute infection, returns to a level somewhat below normal, then starts a slow decrease over time. A marked decrease in $CD4^+$ T cell counts can be observed in some individuals as they develop symptoms. The number of $CD8^+$ T cells (---) rises during primary infection, to return to values just above normal, staying elevated until the final stages of disease. However, the $CD8^+$ cell anti-HIV responses (-.-.-) begin to decrease around the time of symptoms, to decrease steadily as disease progression occurs. ARC, Aids related complex (Modified from ref. 106).

within weeks after acute infection, viral burden is downregulated as an effective immune response develops, which includes antibodies and cytotoxic T cells (144). Cellular immune responses are probably the first effective antiviral activity produced, since CD8⁺ T cell anti-HIV responses have been noted even prior to seroconversion (110). However, despite declines of viral load early in the course of disease, HIV replication does not stop and tends to increase over time (91). Germinal centers in the lymph nodes contain large amounts of HIV-1 in the early and intermediate stages of infection (70).

Three to 4 months after the primary infection, CD4⁺ T cells rise to near normal values, before they start a steady decrease, estimated to be 25-60 cells/ μ l per year (102). The major cause of CD4⁺ T cell death during this stage may be apoptosis, since at this phase of infection the predominant virus detected is the noncytopathic macrophage-tropic strain (130). During this period, CD8⁺ T cell number remains slightly elevated. Virus replication continues, particularly in the lymphoid tissues (142).

When the individual starts to show symptoms of disease, CD4⁺ T cells have usually dropped to around 200 cells/ μ l (adults) and levels of virus in peripheral blood and lymph nodes rise again. A reduction in cytotoxic CD8⁺ T cell responses can also be demonstrated at this stage (110).

Progression to disease. Following infection of HIV-1 the majority of patients experience a long asymptomatic period prior to the development of AIDS, while some individuals become immunosuppressed and develop opportunistic infections rapidly (8). The average time from the onset of HIV-1 infection to clinical AIDS usually ranges from 7 to 10 years. A small group of patients (5%), however, remains clinically asymptomatic despite prolonged infection, and is called long-term nonprogressors. Long-term survivors is a broader definition and includes both asymptomatic and symptomatic patients who generally have been infected for over 10 years.

Long-term nonprogressors have lower levels of intracellular and plasma virus, compared to symptomatic individuals. The virus strain identified is relatively nonvirulent, noncytopathic, macrophage-tropic, and does not replicate in established T-cell lines. Neutralizing antibodies are detected in the blood of these individuals. PBMC from these patients produce type 1 cytokines (interleukin-2 [IL-2] and interferon- β [IFN- β]) and CD8⁺ T cell responses (cytotoxic and suppressing) (152), which are depressed in AIDS patients, remain strong in this group (178). A shift from TH-1 to TH-2 type cytokines (IL-4 and IL-10) occurs with progression to disease (106).

Dynamics of HIV-1 infection. Recently a model of HIV-1 dynamics estimated that in patients with CD4 counts of up to 500, 10^7 to 10^9 virions are produced per day, resulting in

peripheral blood viremia of 10^4 to 10^7 RNA molecules/ml (91, 201). It was also calculated that 2×10^9 $CD4^+$ T cells are destroyed per day and that $> 98\%$ of the plasma virus is produced within recently infected cells (<1 day). Chronically or latently infected cells do not seem to play a major role in the virus turnover, according to this model.

Although such model may be correct for late stage disease, it is yet to be demonstrated if the same holds true for populations of patients with early-stage and asymptomatic disease (121).

Factors in the immune pathogenesis of HIV-1. Multiple factors are responsible for immune deficiency in HIV-1 infection. Genetic background of the host, which determines susceptibility of the cells to HIV replication and the effectiveness of immune response, is important in disease progression. Direct infection of $CD4^+$ cells, alteration in cytokine production and in immune responses (antibody-dependent cell-mediated cytotoxicity [ADCC], cytolytic T lymphocytes [CTL], autoantibodies, and apoptosis), can all play a role in pathogenicity.

Maintenance of an asymptomatic state appears to depend on an adequate production by $CD4^+$ cells of cytokines such as IL-2, needed for $CD8^+$ T cell antiviral activity. The $CD8^+$ T cells will cause suppression of HIV replication and consequently normal $CD4^+$ T cell formation.

The importance of humoral immune responses is uncertain. Neutralizing antibodies are found throughout the entire course of HIV-1 infection. Antibodies would seem to be more useful during the initial phase of infection, when destruction of virus-infected cells (via ADCC) could prevent HIV spread.

The type of virus present in the individual is another important aspect of HIV-1 pathogenesis. Syncytium-inducing (SI) and non-syncytium-inducing (NSI) viruses have been described. The SI phenotype produces cytopathology with formation of multinucleated giant cells or syncytia in PBMC, and can be grown in T lymphoblastoid cell lines. In contrast, NSI viruses do not form syncytia in PBMC, cannot be grown in T lymphoblastoid cell lines, and usually (but not always) grow more slowly than SI variants. Virus isolated from individuals shortly after seroconversion and during asymptomatic infection is predominantly macrophage-tropic and NSI *in vitro*. SI phenotypes are associated with a more advanced clinical stage of HIV disease and with faster depletion of CD4⁺ T cells than viruses of the NSI phenotypes (17, 38, 162, 191).

Animal models of HIV-1 disease. Animal models can be used in the study of the pathogenesis of lentivirus infection and AIDS, in the investigation of perinatal transmission of HIV (159), in the development of vaccines for HIV, and in the assessment of the antiretroviral activity of new drugs. However, an ideal model -- one in which HIV-1 infects an

inexpensive and easily available animal, and produces a disease analogous to AIDS - still does not exist (104).

HIV has been experimentally transmitted to chimpanzees, resulting in a specific antibody response, but these animals do not develop signs of disease. Attempts to produce HIV infection in small animal species (mouse) have been unsuccessful (104).

Lentivirus infection in sheep, goats, horses, cattle, and cats are similar to HIV infection in humans. These viruses are genetically analogous to HIV and share several clinical and immunologic features (105). However, the lentivirus with the most similarities to HIV is the simian immunodeficiency virus (SIV). SIV infection of rhesus monkeys is considered to be the best model for HIV-1 infection of humans (47). The viral genomes of HIV-1 and SIV are closely related, and both viruses infect similar target cells. In addition, SIV disease in rhesus macaques is comparable to human AIDS; SIV infection of adult macaques results in high levels of virus replication, CD4⁺ T cell depletion, and immunosuppression (7, 104).

The severe combined immunodeficient mouse (SCID) transplanted with human peripheral blood lymphocytes has provided a useful model for the study of HIV infection in human T cells in an *in vivo* environment (122). Although these animals do not develop clinical signs of AIDS, the human cells can be infected with HIV, providing a useful model for

quantitation of HIV infection and in the screening of antiretroviral drugs.

Monocyte Infection by HIV-1

Although the main targets of HIV-1 *in vivo* are the CD4⁺ T lymphocytes and tissue macrophages, blood monocytes also express the CD4 surface molecule, consisting of potential targets to the virus. Monocytes are precursors of tissue macrophages, and together with neutrophils, these cells are the main "professional phagocytes" in the body. Monocytes travel unidirectionally from the bone marrow to the tissues, where they differentiate into the long-lived macrophages. In the peripheral blood, monocytes consist of a small fraction of total mononuclear cells (PBMC), usually between 5 and 10% of this population. Infection of these cells could serve as a mechanism of disease dissemination to the tissues, as well as of transmission of HIV-1.

CD14, a 53 to 55 kDa glycoprotein is highly expressed on the surface of mature monocytes, in trace amounts on granulocytes, but not on other hematopoietic cells, including monocyte precursors (179). Differential expression of CD14 is observed in tissue macrophages. Peritoneal macrophages show strong CD14 expression, while alveolar macrophages show weak expression of this molecule (216). CD14, a member of the family of leucine-rich proteins (66), is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (87)

and it functions as a receptor for lipopolysaccharide (LPS) and LPS binding protein (211).

Monocytes/macrophages can serve as a virus reservoir during both early and late stages of HIV-1 disease (96). Macrophage-tropic variants, which are viruses that preferentially infect macrophages over T-cell lines in culture, seem to be transmitted more readily than T-cell tropic viruses in both sexual and vertical transmissions (131, 196). Cord blood monocytes and placental macrophages are more susceptible than adult monocytes to HIV-1 infection in culture (98, 150, 185). Finally, *in vitro* studies suggest that monocytes only become susceptible to HIV-1 during their differentiation into macrophages (173).

However, infection of blood monocytes, *in vivo*, is controversial. Several studies have shown only low levels of HIV-1 present in these cells (123, 125, 169), although one group reported similar levels of infection in monocytes and CD4⁺ T cells by an *in situ* polymerase chain reaction (PCR) assay (9). Many reasons could account for the controversial results. Monocytes comprise a small population of PBMC and selection techniques frequently do not yield highly enriched cell populations. Besides, monocytes differentiate rapidly into macrophages once in culture. Therefore, detection of virus in monocyte populations could be reflecting infection of "contaminating" CD4⁺ T lymphocyte, infection of mature macrophages, or a true monocyte infection (4). Another

confounding factor is that infection of bone marrow stem cells, which are the progenitors of all blood cells including monocytes, is a controversial issue (42, 136, 186).

A previous report demonstrated that nonproliferating quiescent CD4⁺ T lymphocytes are susceptible to virus entry, and that viral DNA synthesis is initiated in these cells with essentially the same efficiency as in stimulated cells, with the difference that only incomplete DNA species are produced in absence of stimulation (214). In this study, spliced RNA transcripts were only detected in stimulated cells, while strong-stop DNA, which is the first region of the HIV-1 genome to be reverse transcribed in the R-U5 region of the LTR, was always demonstrated in quiescent T cells, indicating the presence of input virion. Studies in monocytes derived macrophages (MDM) have subsequently shown that the activation state that coincides with the G1/S phase of the cell cycle, and not DNA synthesis or mitosis itself, is required for establishment of productive HIV-1 infection of these cells. The MDM fraction that lacks proliferative capacity is susceptible to virus entry, although virus does not replicate at this stage (171).

The above results suggest that if blood monocytes are not productively infected, these cells can still be susceptible to viral entry and latent infection, until subjected to mitogen stimulation and consequent proliferation.

Vertical Transmission of HIV-1

The first cases of AIDS in children were described in 1982, approximately a year after the description of AIDS in adults, and a clear connection with maternal infection was then detected (27, 157). The majority of children acquire HIV-1 infection from their seropositive mothers, although infection through contaminated blood products and sexual abuse have also been reported. The highest seroprevalence rates in women are among those who are intravenous drug users or who are sex partners of an HIV-infected man. In the United States, African-American and Hispanic women are at higher risk than Caucasians for infection.

Not all infected pregnant women, however, will transmit HIV-1 to their children. Transmission varies in different parts of the world. In the absence of anti-retroviral therapy, transmission can be as high as 50% to 60% in certain regions of Africa (19, 41, 161), while in Europe it is lower, at 11% to 15% (63, 119). In the United States, 25% to 30% of infants of HIV-1 infected mothers will acquire the infection (52, 160, 209).

HIV-1 may be transmitted to an infant in utero (detection of virus in fetuses as early as 15 weeks old (20), in placentas (98, 175) and in cord blood (185), close to the time of birth or postnatally (breast feeding) (158, 195). Perinatal infection occurs through contact with amniotic fluid, genital

secretions or maternal blood (51). From 30% to 50% of infants are HIV-1 positive by PCR or culture soon after birth, suggesting in utero transmission at some point during gestation (58, 156). Diagnosis of HIV-1 infection in infants cannot be made by detection of HIV antibodies because of placental crossing and persistence of maternal antibodies in the child for up to 18 months. Therefore, ELISA and Western blot tests used for diagnosis in adults are not applicable for children, in whom diagnosis is made by virus culture and/or by PCR.

Maternal transmission of HIV-1 is multifactorial and can be influenced by clinical, obstetrical and virological aspects. Advanced disease stage of the mother (clinical AIDS) with altered immune status, particularly low CD4 count (<200 cells/ μ l) or CD4-to-CD8 ratio, is associated with increased risk for transmission (12, 13, 163). Although the presence of sexually transmitted diseases increases the risk of a woman to become infected by HIV-1, only syphilis and chorioamnionitis were found to be related with higher perinatal transmission rates (163). Mothers who have neutralizing antibodies to HIV-1 seem to be at a reduced risk of infecting their children (95, 100, 165). Macrophage-tropic viruses were reported to be preferentially transmitted to the child (95, 98, 140, 150). Reports have shown maternal transmission of either multiple (81) or selected (2, 207) genotypes. Most likely, heterogeneity of the virus transmitted will depend on the

diversity, viability and tropism of the maternal virus, and susceptibility of neonatal cells.

Studies on the association of levels of plasma p24 antigen (Ag) and transmission are inconsistent. Some reports show that antigenemia correlates with transmission, while others claim that p24 is unrelated to vertical transmission (65, 97, 143, 163). High levels of maternal plasma HIV-1 RNA are associated with increased transmission (49, 64, 95), and the few studies on the association of maternal cell-associated virus levels and vertical transmission suggest a correlation of these factors (45, 155, 202). Because it seems like vertical transmission can occur through both cell free and cell associated HIV-1, it is important to consider viral load in both compartments when analyzing neonatal infectivity risk.

Obstetrical factors are also associated with mother-to-child transmission of the virus. Children born by Caesarean section are at a lower risk to become infected by HIV-1 (55, 63), while birth weight (61, 135) and gestational age (61, 63) also seem to influence the outcome of the children. Pre- and post-term infants have more chances to become infected. Furthermore, studies performed in twins have shown that the first born child is at an increased risk for infection (55, 77).

A large scale, randomized trial, was conducted to evaluate both the efficacy and safety of the antiretroviral drug zidovudine (ZDV) in reducing the risk of maternal-infant

HIV-1 transmission. ZDV, which readily crosses the placenta, is a deoxythymidine analogue that acts by termination of viral DNA production and competition for nucleotides used by the viral RT (106). Infected pregnant women with CD4 counts above 200 cells/ μ l were enrolled in the ACTG (AIDS Clinical Trials Group) protocol 076 between 14 and 34 weeks of gestation. Women either received placebo or oral ZDV during gestation and intravenous intrapartum ZDV, and infants of the treated women received oral ZDV for the first 6 weeks of life. A 67.5% reduction in transmission was detected when comparing transmission in the untreated group (25.5%) to transmission in treated mother-child pairs (8.3%). Hemoglobin levels in infants in the treated group were significantly lower at birth, but by 12 weeks the levels were similar in the 2 groups (36). Although ZDV therapy is efficient in reducing vertical transmission of HIV-1, it is not known which arm of the treatment is responsible for the beneficial effect.

Women with CD4 counts below 200 cells/ μ l, not included in the original ACTG 076 trial, were analyzed in posterior studies. The efficacy of ZDV did not seem to depend on CD4⁺ lymphocyte level, suggesting that women with severe immune depression, who are at highest risk of transmitting, may also benefit from ZDV (117).

Pediatric HIV-1 Infection

As mentioned above, HIV-1 infection in infants occurs mainly through vertical transmission from infected mothers; therefore, the increase in pediatric AIDS cases is due to the growth in the number of HIV-1 infected women of childbearing age (33). Diagnosis of HIV-1 infection in infants can only be made by direct detection of virus in the child. Maternal antibodies are passively acquired by the child and nonspecific signs and symptoms of HIV-1 infection are usually seen at this age. PCR is the most frequently used diagnostic method for its sensitivity and specificity (44, 154). Viral culture and acid-dissociated plasma p24 Ag are also acceptable methods for the diagnosis of HIV-1 in infants (15, 22). Procedures used to detect IgA (113, 114) and IgG (6) HIV-1 specific antibodies in children born to seropositive mothers have been developed, but none guarantees high sensitivity or specificity before 3 to 6 months of age.

Different than in adults, progression to disease is faster in vertically infected infants. HIV-1 infected children show a high incidence of *Pneumocystis carinii* pneumonia (PCP) and encephalopathies. PCP is the most common HIV-associated opportunistic infection in children with AIDS, and according to a 1991 report from the Centers for Disease Control (CDC), 50% of children with AIDS develop PCP at some point of their illness (29). Mortality from PCP, specially in the first months of life is very high, therefore, therapeutic

prophylaxis is essential. In the other hand, although children are prone to develop HIV encephalopathies and myelopathies, pediatric patients rarely present with opportunistic infections of the central nervous system (CNS) (21).

Factors associated with this rapid outcome include the presence of high viral load in children (48, 56), the incompletely developed immunity at this age (109), the timing of HIV-1 transmission (in utero, at birth or postpartum), phenotype/genotype of the transmitted virus, and the absence of neutralizing antibodies. Infants who are infected in utero (HIV-1 PCR or culture positive at birth) usually develop a more rapid increase in viral load, faster loss of CD4⁺ T cells, and earlier progression to AIDS, when compared to children who become infected at birth (48).

Low CD4 counts per age are the most commonly used markers of immune deficiency, indicators of risk of developing opportunistic infections, and response to therapeutic intervention. Lymphocyte subsets vary with age in childhood. In normal children (46) and in uninfected children born to HIV-1 positive mothers (62), median CD4⁺ T cell counts are 3200/mm³ during the first 6 months of life, 3100/mm³ between 7-12 months of age, declining to 2600/mm³ between 13-24 months of age, and 1700/mm³ by 2-6 years of age. Median adult levels of 800-1000 cells/mm³ are reached towards the end of adolescence (46, 62, 94).

CD4⁺ T cells can be further divided into "naive" and "memory" cell populations, phenotypically divided by reaction with monoclonal antibodies (MAbs) directed against certain cell surface molecules as the CD45RA⁺ and CD45RO⁺ subsets, respectively (164). In normal neonates, more than 90% of CD4⁺ T cells in the peripheral blood express CD45RA and less than 10% express the memory phenotype, declining to around 37% in adults (94). In adults, HIV-1 preferentially infects the CD45RO memory T cells (168). In children >2 years of age the memory subtype is also preferentially infected, while in infants, both cell types seem to be susceptible to HIV-1 (181).

My project was aimed into a better understanding of the immunopathogenesis of HIV-1 maternal transmission and pediatric infection, specially during the first months of life, as infants progress from acute to chronic HIV-1 disease. The specific aims were: 1. To determine the main target cell of HIV-1 in mother and child infection; 2. To determine an association among pediatric HIV-1 proviral copy number, timing of infection and progression of disease; and 3. To evaluate the correlation between maternal HIV-1 proviral copy number and transmission, in the absence or presence of ZDV therapy.

This study should add valuable information for therapeutic approaches and drug development strategies.

CHAPTER 2 MONOCYTE SELECTION TECHNIQUE

Introduction

Monocytes traffic unidirectionally from the bone marrow through the blood to tissues, where the cells differentiate into macrophages. Peripheral monocytes are frequently targeted for studies of human cells of the monocyte/macrophage lineage because of their accessibility through blood sampling. The techniques developed to isolate monocytes from other peripheral blood mononuclear cells (PBMC) rely on the adherent characteristics of monocytes in culture, their size differences from other PBMC, or their expression of monocyte-restricted cell surface proteins such as CD14 (43, 200, 213, 215, 216). Physical methods used for monocyte selection require large volumes (50 ml or more) of blood, which hinder their application in pediatric studies involving infants and young children. Monocytes compose only 5 to 20% of total PBMC in contrast to lymphocytes, which constitute the major population of PBMC. Adherence or elutriation is generally efficient in depleting monocytes from PBMC but often does not generate an enriched monocyte population significantly depleted of lymphocytes (93,200).

We developed a technique that utilizes magnetic microspheres, or beads, in combination with monoclonal antibodies (MAbs) targeted at CD14 or CD4 to select highly enriched populations of monocytes and CD4⁺ T cells. CD14, a receptor for lipopolysaccharide, is a surface glycoprotein with a size of 55 kDa expressed on cells of monocyte/macrophage lineage (24, 87, 179, 211, 216). CD4 is expressed on both T helper cells and monocytes. A sensitive molecular strategy based on PCR amplification and detection of T-cell receptor (TCR) gene rearrangements demonstrated the effectiveness and reproducibility of the technique to yield viable monocyte populations which contain fewer than 2% contaminating T lymphocytes. The technique is effective even when isolating cells from small volumes of blood.

Materials and Methods

Cell samples. Peripheral blood (5 to 10 ml, maximum of 2 ml/Kg of body weight) from 17 children and adult volunteer blood donors was collected in heparinized tubes according to a protocol approved by the Institutional Review Board of the University of Florida. Samples were diluted 2 to 1 (vol/vol) in Hank's balanced salt solution (HBSS) without calcium and magnesium (GIBCO BRL, Grand Island, NY) plus 20% fetal bovine serum (FBS) (GIBCO BRL). PBMC were collected by Ficoll-Hypaque density centrifugation (Histopaque 1077; Sigma Diagnostics, St. Louis, MO), as previously described (183). The mononuclear

cells were collected and washed twice in HBSS plus 20% FBS. All cell counts were carried out by using a hemocytometer. Cell viability was always >98%, as determined by trypan blue exclusion. The cell lines used for control experiments included Jurkat, a human T-cell line (203), and HeLa, a human nonlymphoid cell line (167). The cell lines were obtained from the AIDS Research and Reference Reagent Program: HeLa from Richard Axel and Jurkat clone E6-1 from the American Type Culture Collection.

Immunomagnetic separation. PBMC (10^7 cells per ml of HBSS plus 20% FBS) were incubated at 4°C for 30 min with an anti-CD14 mouse MAb, MY4 (125 µg/ml) (Coulter Immunology, Hialeah, FL), diluted 1 to 100. PBMC were also incubated with a mouse immunoglobulin G (IgG) antibody, MsIgG (1,000 µg/ml) (Coulter Immunology), or with no antibody at a similar dilution. The cells were washed twice with cold phosphate-buffered saline (PBS) plus 10% FBS (GIBCO BRL) and resuspended at the original concentration.

Immunomagnetic microspheres, or beads, coated with sheep anti-mouse IgG (Dynabeads M450; Dynal, Oslo, Norway) were washed in PBS, counted by using a hemocytometer, and resuspended in PBS plus 10% FBS at a final volume equal to that of the target cells. The number of beads added to deplete the PBMC of monocytes was calculated by using a ratio of 10 beads per target cell. The estimated frequency of CD14⁺ cells

was based on the subjects' complete blood counts with differentials as determined by a Coulter Counter.

Beads and antibody-coated mononuclear cells were mixed in polypropylene round-bottom tubes (12 by 75 mm, Falcon 2005; Becton Dickson Labware, Lincoln Park, NJ) and incubated with gentle rotation at 4°C for 30 min. Monocytes with beads were separated from the CD14-negative fraction by placing the polypropylene tubes containing the cell suspension in the presence of a Dynal MPC-1 magnet. Cells bound to beads adhered to the tube in the magnetic field while nonadherent cells were gently removed by pipetting. Immunomagnetic selection and washing were repeated four to five times or until, as determined by light microscopy, all beads were removed from the suspension.

CD4⁺ T cells were selected from the CD14-depleted fraction (resuspended at 10⁷ cells per ml) by incubation with an anti-CD4 mouse MAb, T4 (500 µg/ml) (Coulter Immunology), at a 1 to 100 dilution. Immunomagnetic beads were used at a ratio of 10 beads per target cell, estimated as 50% of the CD14-depleted cells.

Monocyte selection by adherence to plastic. PBMC (4 X 10⁶ to 6 X 10⁶ cells per ml) were resuspended in RPMI 1640 medium (GIBCO BRL) supplemented with 20% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 U of penicillin per ml, 50 mg of streptomycin per ml, and 10% freshly pooled human serotype AB serum. Cell suspensions were incubated for 1 h at 37°C under

5% CO₂ in 100 cm² tissue culture plates (Falcon 3003; Becton Dickson Labware). Non-adherent cells were removed by aspiration with a Pasteur pipette. Adherent cells were washed three times with cold medium and dislodged from the plates with a plastic cell scraper (Costar, Cambridge, MA.)

Flow cytometry analysis. Unfractionated PBMC and aliquots of cells remaining after immunomagnetic selection were prepared for one- and for two-color flow cytometry analysis as previously described (183). The cells were stained with fluorescein isothiocyanate (FITC)-conjugated mouse MAbs (anti-CD3, Leu-4, 100 µg/ml; anti-CD4, Leu-3a, 3 µg/ml; anti-CD14, Leu-M3, 25 µg/ml; anti-CD19, Leu-12, 25 µg/ml; and anti-CD8, Leu-2a, 12.5 µg/ml [Becton Dickinson Immunocytometry Systems, San Jose, CA]). The numbers and percentages of CD3⁺CD4⁺ T cells and CD4⁺CD14⁺ monocytes within the unfractionated PBMC were determined by two-color analysis with phycoerythrin-conjugated Leu-3a (10 µg/ml) and FITC-conjugated Leu-M3 and Leu-4 (Becton Dickinson Immunocytometry Systems). Cell samples were incubated with the MAbs at 4°C for 30 min in the dark. For two-color staining, the cells were first incubated with the FITC-conjugated MAb, washed, and then incubated at 4°C for 30 min with the phycoerythrin-conjugated MAb. Controls consisted of cells stained with isotype-matched phycoerythrin- or FITC-conjugated mouse IgG (50 µg/ml) (Becton Dickinson Immunocytometry Systems). Cells were washed (0.01 M PBS, 0.02% sodium azide, 10% fetal calf serum), fixed (0.01 M PBS, 0.02%

sodium azide, 1% paraformaldehyde), and stored at 4°C in the dark until analysis. Cell fluorescence was analyzed by using a Becton Dickinson FACScan flow cytometer. Lymphocyte and monocyte populations were defined on the basis of differences in forward angle and side scatter of the two populations, as previously described (74, 183). Dual-color analysis was carried out by using a compensation network. The fluorescence from 10⁴ cells per sample was quantified.

DNA extraction. Cells were resuspended in 1 ml of lysis buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 2% sodium dodecyl sulfate [SDS], 100 µg of proteinase K per ml). After overnight incubation at 37°C, DNA was extracted by using the G NOME DNA isolation kit (BIO 101, Inc., La Jolla, CA) according to the manufacturer's protocol. The DNA concentration was calculated from spectrophotometric readings of the samples with a Beckman model DU 640 spectrophotometer.

PCR. Primers specific for the V_β8 family of TCR were V_β8-forward (5'-AACGTTCGATAGATGATTCAGGGATGCCC-3') and J_β1.2-reverse (5'-TACAACGGTTAACCTGGT-3'), as previously described (182). Oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer in the DNA synthesis core facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. The amplified products were 180 bp in length. β-Actin sequences were amplified with a forward primer (5'-GAAACTACCTTCAACTCCATCATG-3') and a reverse primer (5'-CTAGAAGCATTGCGGTGGAC-3') (Clontech, Palo Alto, CA). The

products amplified with the β -actin primers were 350 bp in length.

Amplifications were performed in a total volume of 50 μ l containing 0.1 and 1 ng of DNA for β -actin reactions or 0.1, 1, 10, 50, and 100 ng DNA for V β 8-J β 1.2 reactions, 200 μ M each deoxynucleoside triphosphate, PCR buffer (50 mM KCl, 1.75 mM MgCl₂, 100 μ g of nuclease-free bovine serum albumin [BSA], 20 mM Tris [pH 8.4]), 1 μ M each primer, and 2.5 U of Taq DNA polymerase (Pharmacia). The samples were covered with mineral oil, and amplification was carried out in a 48-well automated thermal cycler (Perkin Elmer Cetus).

V β 8-J β 1.2 amplification involved 1 cycle of denaturation (94°C for 5 min), 35 cycles of amplification (denaturation for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 2 min at 72°C), and 1 cycle of extension (72°C for 10 min). β -Actin amplification involved 1 cycle of denaturation (94°C for 5 min), 25 cycles of amplification (94°C for 30s, 60°C for 30s, and 72°C for 30s), and 1 cycle of extension (72°C for 10 min).

Southern blot analysis. Amplification products in a volume of 10 μ l were electrophoresed in 1.2% agarose gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH), according to the method of Southern (184). Double-stranded DNA probes used to detect TCR recombinants, and β -actin sequences (182) were labelled by random priming with [³²P]dATP (Du Pont, Boston, MA) to a specific activity of at least 10⁹ cpm/ μ g of DNA and placed over a Sephadex G-50

(Pharmacia, Uppsala, Sweden) spun column to retain the free nucleotides. Filters were prehybridized for 2 h in hybridization buffer (1 mM EDTA [pH 8.0], 0.5 M NaPO₄ [pH 7.2], 7% SDS, 1% BSA), hybridized for 16 h with 10⁶ cpm of radiolabelled probe per ml of buffer, and washed for 1h, as previously described (83, 182). Hybridization and washes were carried at 55°C for β -actin and at 50°C for V β 8 analysis. Membranes were exposed at -80°C to Fuji medical X-ray film.

Electron microscopy and histologic analysis of monocytes.

Samples (10⁵ cells per sample) were centrifuged in a cytospin centrifuge (model SCA-0031; Shandon Southern Products Ltd., Runcorn, Cheshire, England) and fixed in citrate-acetone-formaldehyde fixative (Sigma Diagnostics). Cells were then stained with α -naphthyl butyrate esterase (Sigma Diagnostics), according to the manufacturer's instructions.

Cells (2 X 10⁶ cells per sample) were pelleted and fixed in 2% glutaraldehyde in PBS (pH 7.3) for 1 h. All subsequent steps were performed at the electron microscopy core laboratory of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Briefly, cells were washed in buffer, postfixed for 30 min in 1% OsO₄, dehydrated in an ethanol series, and embedded in Spurr's epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined by using a Hitachi model SH-7000 electron microscope.

Statistical analysis. Statistical analysis of cell yields from blood volumes of less than 6 ml compared with blood volumes of more than 6 ml was carried out by using the Student t test. Values are expressed as the means \pm standard deviations.

Results

Cell yield and efficiency by flow cytometry analysis. PBMC were isolated from blood samples, with volumes ranging from 3 to 20 ml. The number of PBMC collected from the 17 individuals studied ranged from fewer than 1×10^6 to 10×10^6 cells per ml depending on the age of the subject. Dual-color flow cytometry analysis of PBMC indicated that more than 85% of CD14⁺ blood monocytes also express CD4 (data not shown). Therefore, CD14⁺ cells were selected before isolation of the CD4⁺ T lymphocytes (Fig. 2.1). Monocytes were selected from the total PBMC by using an anti-CD14 MAb (MY4) and magnetic beads. Control experiments consisted of PBMC incubated with MsIgG or without murine antibody. Immunoaffinity magnetic beads were then added as described in Materials and Methods. CD4⁺ T lymphocytes were selected from the CD14-depleted fraction with an anti-CD4 MAb (T4) and magnetic beads.

Cell yields from the selected fractions were calculated on the basis of flow cytometric analysis of the number of monocytes or CD4⁺ T cells in unfractionated PBMC (Table 2.1). The number of cells obtained by immunomagnetic bead selection

Figure 2.1. Schematic representation of the PBMC separation procedure steps to select CD4⁺ T lymphocytes and CD14⁺ monocytes. PBMC were separated from heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. CD14⁺ monocytes were selected by incubating the cells first with an anti-CD14 MAb and then with magnetic beads conjugated to a sheep anti-mouse antibody. The CD14⁺ cells were separated from the CD14-negative cells by placing the cell mixture in the presence of a magnetic field. The washed, positively selected cells were placed in lysis buffer for DNA extraction followed by PCR amplification with TCR and actin primer pairs. The CD14-negative cell fraction was incubated with an anti-CD4 MAb and then with magnetic beads conjugated with sheep anti-mouse antibodies. The CD4⁺ cells were selected in the presence of a magnet and washed, and the DNA was extracted and amplified with TCR and actin primers.

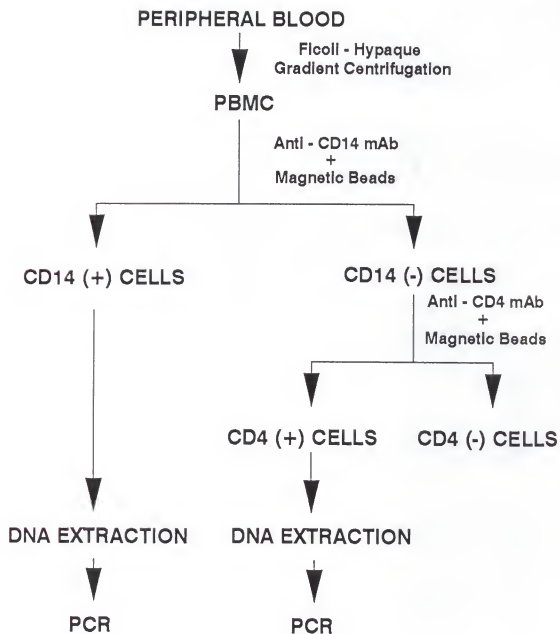


Table 2.1. Average cell yield of CD14⁺ monocytes and CD4⁺ T cells from different blood volumes^a

Blood No. of vol (ml) samples	Avg. no. of PBMC/ ml of blood	Avg. no. of CD14 ⁺ cells		Avg. no. of CD4 ⁺ T cells	
		Expected ^b	Selected ^c	Expected ^b	Selected ^c
<6	8 (4.8 ± 3.4) X 10 ⁶	(8.0 ± 2.5) X 10 ⁵	(6.0 ± 3.0) X 10 ⁵	75.6 ± (16.7 ± 8.9) X 10 ⁵	5.1 ± (6.7 ± 3.6) X 10 ⁵
6	9 (3.2 ± 2.6) X 10 ⁶	(6.1 ± 3.8) X 10 ⁵	(4.3 ± 2.4) X 10 ⁵	80.5 ± (9.7 ± 5.5) X 10 ⁵	2.9 ± (4.2 ± 4.3) X 10 ⁵
					41.1 ± 14.2 36.1 ± 15.9

^a Values represent the means ± standard deviations for the individual blood samples examined

^b Average number of CD14⁺ monocytes or CD4⁺ T cells per milliliter of blood as determined by fluorescence-activated cell sorter analysis

^c Average number of cells of each type selected per milliliter of blood by using immunomagnetic beads

^d Percent yield is calculated from the number of selected cells/number of expected cells X 100 for each experiment; when the yields were compared on the basis of blood volumes for each cell type, the results were not statistically significant (p>0.05)

was determined with a hemocytometer to count the cell-bead aggregates. The results of flow cytometry analysis of unfractionated PBMC indicated an average of $(8.0 \pm 2.5) \times 10^5$ CD14⁺ monocytes per ml of blood from samples containing less than 6 ml of blood. Following immunomagnetic selection, $(6.0 \pm 3.0) \times 10^5$ monocytes per ml were recovered, a number which represents an average yield of 75.6% of the peripheral monocytes expressing CD14. CD14⁺ monocytes selected from 6 to 20 ml of blood resulted in a recovery of 80.5% of the potential blood monocytes. The recovery of monocytes from blood volumes less than 6 ml was as efficient as monocyte recovery from larger volumes of blood (≥ 6 ml). There was no evidence of cell-bead aggregates when the PBMC incubated with MsIgG or the controls with no antibody were examined by light microscopy. These results indicate that the use of an anti-CD14 MAb is required for immunomagnetic selection of monocytes. Nonspecific binding of the control antibody to monocytes did not result in significant binding of the beads.

The overall yield of the selection of CD4⁺ T cells from PBMC was 41.1% for small blood volumes and 36.6% for blood volumes of more than 6 ml. The efficiency of selection of CD4⁺ T cells from the CD14-depleted cell fraction was greater than 75% (data not shown). The loss of CD4⁺ T cells appears to be a result of the extensive manipulation and cell washes required for the two rounds of bead selection, because cell losses within the CD19⁺ B cells and CD8⁺ T cells in the CD4-

depleted cell fraction were similar (data not shown). The yields of CD14⁺ monocytes or CD4⁺ T cells were not statistically different when small and large blood volumes were used.

On the basis of the sensitivity of flow cytometry analysis of the depleted fractions, greater than 95% of the monocytes were depleted from the PBMC fractions following immunomagnetic bead selection with the CD14 MAb (Fig. 2.2). There was no evidence of monocyte depletion following incubation of PBMC with MsIgG or beads alone (data not shown). Fewer than 5% residual CD4⁺ T cells were detected following selection with the anti-CD4 MAb (Fig. 2.2).

Electron microscopy evaluation of CD14⁺ monocyte and CD4⁺ T-cell enrichment. Morphologic analysis of unfractionated PBMC and immunoselected CD14⁺ monocytes or CD4⁺ T lymphocytes by light microscopy indicated greater than 99% enrichment for the selected cell type (data not shown). No contaminating lymphocytes, either B cells or T cells, were detectable within the CD14⁺ cell fraction. Furthermore, by cyto-spin centrifugation and staining with α -naphthyl butyrate esterase, the bead-selected CD14⁺ cell population showed a monocyte morphology. However, the esterase staining pattern of the bead-selected cells was atypical compared with that of the stained monocytes within the PBMC (data not shown).

To more precisely examine the CD14⁺ cells, electron microscopy was chosen as an additional method of monocyte

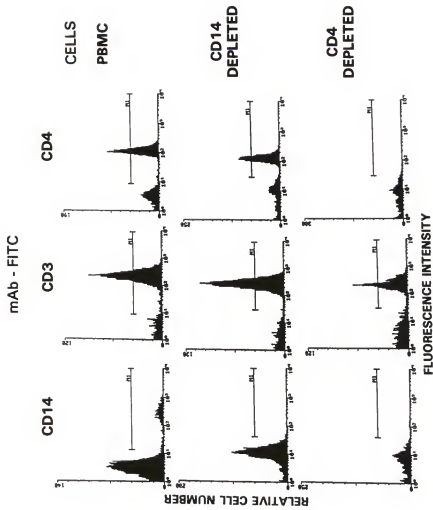


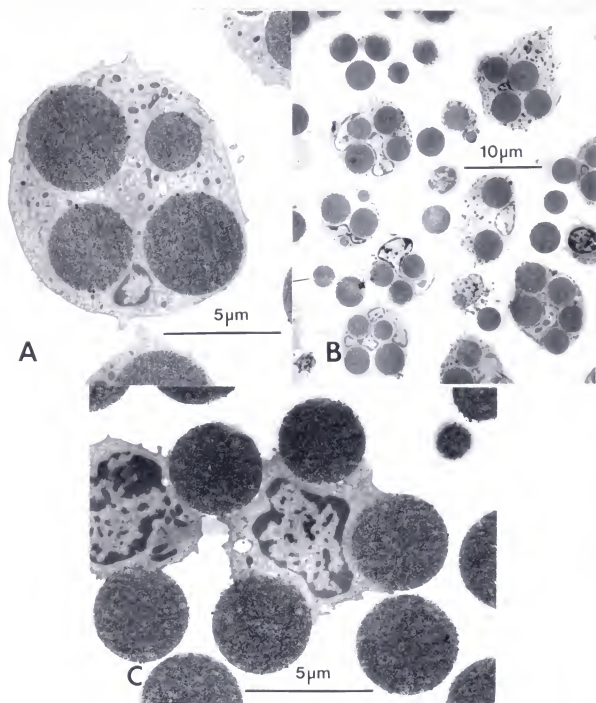
Figure 2.2. Flow cytometry analysis of CD14⁺, CD3⁺, or CD4⁺ PBMC populations stained with FITC-conjugated mouse MAb anti-CD14 (Leu-M3), anti-CD3 (Leu-4), or anti-CD4 (Leu-3a), respectively.

identification. CD14⁺ monocytes and CD4⁺ T cells within unfractionated PBMC were easily distinguishable by electron microscopy on the basis of their morphologies (data not shown). Following magnetic selection, the CD4⁺ T-cell fraction was enriched for lymphocytes with magnetic beads attached to the surface of these cells (Fig. 2.3C). The selected CD14⁺ fraction was highly enriched for monocytes with no evidence of contaminating lymphocytes. Magnetic beads appeared mostly internalized within the monocytes rather than on the surface, which indicates active phagocytosis of the CD14-magnetic bead complex (Fig. 2.3A and B). Assessment of cell viability by trypan blue exclusion after 24 h of culture indicated that phagocytosis of the beads did not impair viability.

Assessment of monocyte enrichment by molecular analysis.

A primary goal of the selection strategy was to achieve a monocyte population with more than 99% depletion of T lymphocytes, which is beyond the detection sensitivity of morphologic or flow cytometric analysis. A more sensitive method by using PCR was developed to assess the extent of residual T lymphocytes within the CD14⁺ monocyte fraction. DNA from both CD14⁺ and CD4⁺ cell fractions was amplified by using TCR primers to detect recombination of variable, diversity, and joining gene segments which occur exclusively in T lymphocytes. The TCR V_β8 family was used as a marker for T cells for two reasons: (1) V_β8 is involved in 3 to 26% of TCR variable, diversity, and joining gene segment recombinations

Figure 2.3. Transmission electron microscopy of monocytes and CD4⁺ T cells selected from PBMC by using immunomagnetic beads. Monocytes coated with an anti-CD14 MAb, MY4, were selected after incubation with magnetic beads. The beads can be seen within the cells (A and B). (C) An anti-CD4 MAb, T4, was added to the CD14-depleted cells, and CD4⁺ T cells were selected following incubation with magnetic beads. The beads are seen attached to the surface of the cells. Magnifications X 10,000 (A), X 3,750 (B), and X 15,000 (C).



in $CD4^+$ T lymphocytes and represents one of the more frequently rearranged TCR gene families in peripheral blood T cells (84, 148), and (2) there is a T-cell line, Jurkat, with a rearranged $V_{\beta}8-J_{\beta}1.2$ TCR, which provides an important positive control for quantitation.

The sensitivity of the primers to detect small numbers of rearranged TCRs within cellular DNA was determined in control experiments using serial dilutions of DNA from Jurkat cells. The 180-bp $V_{\beta}8$ TCR product was consistently detected at the level of a single Jurkat T cell (0.01 ng of input DNA) (Fig. 2.4A). The amount of template DNA was verified by amplification of serial dilutions of Jurkat DNA with primer pairs for β -actin, which also detected a single cell (Fig. 2.4B). The sensitivity for detection of a single cell by amplification was the same when serial 10-fold dilutions of Jurkat cells were mixed with reciprocal dilutions of HeLa cells, a nonlymphoid cell line without TCR rearrangements, prior to DNA extraction (data not shown). On the basis of the results of these experiments, each subsequent PCR amplification included a standard curve by using serial dilutions of Jurkat DNA.

The frequency of $V_{\beta}8$ T cells within the monocyte fraction was compared with the number of $CD4^+$ T cells which had $V_{\beta}8$ rearrangements within the $CD4^+$ T-cell fraction from the same individual. Equivalent amounts of template DNA from the different populations were verified by amplification with

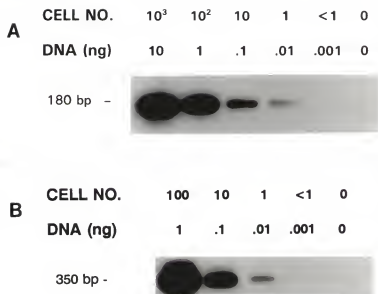


Figure 2.4. Amplification of serial dilutions of DNA obtained from Jurkat T cells. The number of cells equivalent to the amount of input DNA was determined in preliminary experiments. Serial dilutions of Jurkat DNA, equivalent to 10^3 to less than 1 cell, were amplified by using forward and reverse primers for either $V_{\beta}8$ and $J_{\beta}1.2$ TCR sequences (A) or β -actin (B). The amplified products were electrophoresed in 1.2% agarose gels and transferred to Nytran membranes. Double-stranded probes to detect the 180 bp TCR product or the 350 bp actin product were labelled by random priming with [32 P] dATP, washed, and exposed to X-ray film.

primers for β -actin (Fig. 2.5). When the amplified products of serial dilutions of DNA from $CD4^+$ T cells were analyzed, $V_{\beta}8$ sequences were detected in the equivalent of 10^2 T cells (Fig. 2.5A). The intensity of the product was equivalent to the signal from 10 cells in the standard curve (Fig. 2.4A) (densitometric analysis not shown). The results indicate that approximately 10% of the T cells in this individual had rearranged $V_{\beta}8$ TCR genes. When 5,000 $CD14^+$ cells were examined, 1 cell with a $V_{\beta}8$ TCR rearrangement was detected (Fig. 2.5A). If $V_{\beta}8$ T cells represent 10% of the total T cells in this individual, then there are about 10 residual T cells per 5,000 $CD14^+$ monocytes, which indicates 99.8% purity of the monocytes. In a second individual, approximately 10 rearranged $V_{\beta}8$ TCR genes were detectable in 1,000 $CD4^+$ T cells (Fig. 2.5B), indicating that $V_{\beta}8$ T cells represent about 1% of the $CD4^+$ T cells in this individual. In contrast, T cells with a rearranged $V_{\beta}8$ TCR were not detected in DNA from 5×10^3 monocytes (Fig. 2.5B). If as many as 100 T cells were present in 5,000 monocytes (2%), the selected monocytes were 98% enriched for $CD14^+$ cells. Cell separations from 17 different individuals consistently produced $CD14^+$ monocyte populations which contained as few as 0.2% and no more than 2% detectable T lymphocytes, even when small volumes of peripheral blood were used.

Immunomagnetic selection of monocytes was compared with selection of monocytes by adherence to plastic. Both

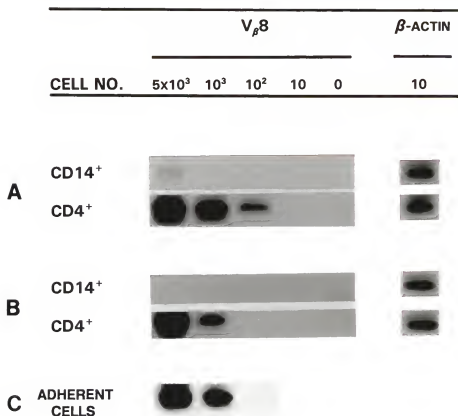


Figure 2.5. Amplification of DNA obtained from PBMC selected by either immunomagnetic beads or adherence to plastic. (A and B) Serial dilutions of DNA equivalent to 5×10^3 cells were obtained from CD14⁺ monocytes and CD4⁺ T lymphocytes selected from 2 separate individuals by using immunoaffinity magnetic beads. (C) Amplification of DNA from serial dilutions of monocytes collected by adherence to plastic. DNA from each cell type was amplified with $V_{\beta}8$ and $J_{\beta}1.2$ TCR primer pairs. The products were electrophoresed in 1.2% agarose gels and transferred to Nytran membranes. The DNA equivalent of 10 cells (0.1 ng of DNA) from each cell type was amplified with primers for β -actin. DNA was amplified by using the $V_{\beta}8$ forward and reverse primer pairs. Double-stranded probes to

techniques effectively depleted monocytes from the larger population of PBMC as determined by flow cytometry analysis (data not shown). However, the level of detectable T cells in monocytes collected by adherence was at least 10-fold higher than in monocytes selected by immunomagnetic techniques (Fig. 2.5C).

Discussion

Studies of human monocyte/macrophage lineage cells have been hampered by a lack of techniques to verify the relative purity of highly enriched blood monocyte populations. This is a particular problem in pediatric studies that are restricted to using small volumes of blood. Assays of monocyte-macrophage function, tropism studies of infectious pathogens such as human immunodeficiency virus, and immunologic assessment of CD4⁺ T cell-to-monocyte interactions would benefit from a simple technique which physically separates the two PBMC populations without affecting viability. Methods used to separate CD4⁺ T cells and monocytes must compensate for expression of the CD4 molecule on both cell types. Our selection scheme results in highly enriched populations of monocytes and CD4⁺ T cells depleted of other contaminating cell types.

Immunomagnetic monocyte selection results in a highly enriched population of CD14⁺ monocytes with more than 98% depletion of T cells. Compared with other methods for monocyte

selection, such as adherence to plastic, the immunomagnetic selection method reduces the number of residual T cells more than 10-fold. Immunomagnetic selection is effective for depletion or for enrichment of targeted lymphocyte populations from human blood and bone marrow (74, 103). Using this procedure, we were able to obtain more than 75% of the expected monocyte population, even from blood volumes of less than 6 ml. However, the extensive manipulations required for each selection step result in cell loss. When this technique is used, the selection strategy should consider the targeted cell population in order to optimize cell yields.

Techniques to verify the extent of enrichment for cell populations that are selected by the immunomagnetic bead method have limitations. Strategies are available to dissociate the magnetic beads from lymphocyte populations (149), although antibody binding to the differentiation marker on the surface of the selected target cell hinders subsequent analysis by flow cytometry. The dissociation of beads can not be applied to immunoselected CD14⁺ monocytes because we found that the CD14-antibody-bead complex becomes internalized within minutes of binding to the cell. Verification of monocyte purity by flow cytometry techniques is further hampered by changes in the light scatter of the monocytes containing the bead complex. Morphologic analysis using light or electron microscopy can be useful to ensure monocyte

enrichment but is not a sufficiently sensitive method to determine the depletion of contaminating T cells.

The PCR-based strategy proved to be a sensitive and reproducible method to determine the level of residual T cells within the monocyte fraction. The effectiveness of the molecular assay is enhanced by several factors. First, the detection of the rearranged TCR can be standardized and the number of TCR rearrangement can be quantified. TCR rearrangements in DNA provide a direct assessment of the number of T cells and minimize variability from determining expression of $V_{\beta}8$ TCR in RNA of blood T cells from different individuals (190, 199). Second, rearrangements in $V_{\beta}8$ are found frequently in circulating blood T cells in most individuals (84, 148). The comparison of the amplification of $V_{\beta}8$ TCR from monocyte DNA and DNA from T cells from the same individual served as an internal control for sensitivity. Amplified $V_{\beta}8$ TCR sequences were at least 100-fold higher in the $CD4^+$ T-cell fractions than in the corresponding monocyte population.

Internalization of the CD14-bead complex does not affect monocyte viability. In addition, MAbs directed at CD14 can result in monocyte activation (174). Our results indicate that anti-CD14 antibody is required for binding and phagocytosis. Whether internalization of the magnetic bead complex would limit the application of the immunomagnetic selection technique to some studies of macrophage function needs to be

evaluated. We have found that bead-selected monocytes can be used for studies of human immunodeficiency virus type 1. Immunomagnetic selection for monocytes in conjunction with a sensitive molecular assay for detection of residual T lymphocytes provide a strategy for obtaining cells of the monocyte/macrophage lineage from children and adults.

CHAPTER 3 VERTICAL TRANSMISSION OF HIV-1

Introduction

In the absence of antiviral intervention, about 25% to 30% of HIV-1 infected pregnant women in the United States transmits the virus to her infant. Multiple factors increase the risk of HIV-1 transmission from mother-to-child (151). Maternal immunity, biological characteristics of the infecting virus, obstetrical factors related to the delivery, and levels of maternal viral load can influence the probability that an infant born to an HIV-infected mother will ultimately become infected (12, 35, 49, 64, 77, 100, 119, 163). The precise contribution by each factor or combinations of factors to ultimate maternal-infant HIV-1 transmission is not clear, although maternal viral factors are important (52). For example, a number of studies demonstrate a direct association between levels of maternal virus, as measured by p24 antigenemia, quantitative viral culture, viral DNA, or quantitative viral RNA, and an increased probability for infection of the infant (49, 65, 95, 97, 202). In addition, drug therapy targeted at the virus produces a significant decrease in vertical transmission of HIV-1. A large scale, blinded, randomized, placebo-controlled clinical trial (ACTG

protocol 076) demonstrated that pediatric HIV-1 infection can be reduced by as much as 67% when HIV-infected mothers and their infants were treated with zidovudine (ZDV) (36).

Although the reduction in perinatal HIV-1 infection by ZDV treatment is striking, the mechanism by which ZDV reduces maternal transmission is unknown. Both cell-free and cell-associated virus have been implicated in maternal-infant transmission of HIV-1 (155, 163), so one possibility is that ZDV administered according to the ACTG 076 protocol reduces maternal plasma virus levels. However, preliminary reports suggest that ZDV-associated reduction in transmission may be independent of levels of maternal viremia (64). ZDV treatment reduces levels of HIV-1 provirus in peripheral blood cells in approximately 50% of infected adults (108), which raises an alternative possibility that the ZDV effect on maternal transmission could be reduction of cell-associated virus in mothers. We and others have shown a close genetic relationship between viruses within peripheral blood mononuclear cells (PBMC) of mothers and their newborns, suggesting that transmission of cell-associated virus is one mechanism for pediatric infection and a potential target for the effects of antiviral drugs (81, 207).

We tested this hypothesis by measuring HIV-1 DNA copies within PBMC of a group of untreated HIV-infected pregnant women and a group of infected pregnant women who received ZDV to reduce transmission. The two groups of women were similar

with respect to age, race, disease stage, blood CD4⁺ T cell counts, and mode of delivery. Transmission was reduced to 10% among infants in the ZDV-treated group, indicating that antiviral therapy was effective. However, there was no difference between groups of treated and untreated women in levels of HIV-1 DNA copies in CD4⁺ T cells. Our results provide evidence that the mechanism by which ZDV reduces pediatric HIV-1 infection is independent of maternal cell associated virus levels. It is possible that ZDV therapy alters the infectivity of the transmitted virus or alters infectibility of susceptible pediatric cells.

Materials and Methods

Subjects. Study subjects were 42, HIV-infected pregnant women who were enrolled between October, 1989 and December, 1995 in an ongoing study of HIV-1 genetic variability according to a protocol approved by the Institutional Review Board of the University of Florida. Forty-one women were enrolled at the University of Florida (UF) in Gainesville, FL and one at the University of South Florida (USF) at Tampa, FL. Nineteen of these women in the study and their neonates received ZDV according to ACTG protocol 076, which was initiated in February, 1994 (36). Twenty-three women received no form of antiretroviral therapy, including 3 women who were offered but refused ZDV therapy. Blood samples were obtained within 24 hours of delivery, except for 2 samples obtained

from untreated mothers which were drawn about 3 months after delivery. Informed consent was obtained from each subject enrolled. Clinical data collected prospectively included the age, race, mode of delivery, obstetrical complications, weight and gestational age of the child, maternal blood CD4⁺ T cell counts, CD4 to CD8 T cell ratio, and CDC (Centers for Disease Control) stage of maternal HIV infections (30). T-cell subsets were determined by flow cytometry analysis at the Clinical Laboratory at Shands Hospital, at the University of Florida.

Transmission status of the mother was determined based on results of subsequent evaluations of the infants. Mothers were classified as transmitting if HIV-1 was detected by polymerase chain reaction (PCR) amplification of PBMC DNA obtained from the infant on at least 2 occasions by 6 months of age (180). Mothers were classified as non-transmitters if results of the PCR analysis of the infant's PBMC DNA was negative at 6 months of age and the child seroreverted to an HIV antibody negative status.

Lymphocyte isolation. Ten milliliters of maternal peripheral blood were collected in acid citrate dextran (ACD) tubes and processed within 24 hours. Following separation of the blood samples into cell and plasma fractions, PBMC were isolated using Ficoll-Hypaque (Histopaque 1077; Sigma Diagnostics, St. Louis, MO) density centrifugation.

CD4⁺ T cells were separated from the PBMC using immunoaffinity magnetic microspheres (Dynabeads M450; Dynal,

Oslo, Norway) according to our previously described methods (4). Cells were resuspended in 1 ml lysis buffer (10 mM Tris at pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% SDS, 100 μ g proteinase K), and incubated at 37°C overnight. DNA was extracted using the G NOME DNA isolation kit (BIO 101, Inc., La Jolla, CA), as suggested by the manufacturer. DNA concentration was calculated by spectrophotometric reading (Beckman DU 640).

PCR analysis. Oligonucleotide primers for amplification of *env* region were forward (5'-GCCACACATGCCTGTGTACCCACA-3') and reverse (5'-CTTCTCCAATTGTCCCTCATA-3'), located at nucleotides 6464 to 6486 and 7693 to 7713, respectively, in the HIV_{LAI} genome. A second set of primers was located in the *gag-pol* region and consisted of forward (5'-GACCAGCAGCTACACTAGAAGA-3') and reverse (5'-TGCGGGATGTGGTATTC-3') primers located at nucleotides 1802 to 1823 and 2863 to 2879, respectively. Primers were synthesized on an Applied Biosystems DNA synthesizer in the DNA synthesis core facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. β -actin primers were obtained from Clontech, Palo Alto, CA. Sensitivity of each set of primers was at the level of 1 to 5 copies (4, 181).

DNA concentrations were 1 and 10 ng for actin amplifications and 100 to 1500 ng of patient DNA for *env* and *gag-pol* amplifications. DNA from the 8E5 cell line, a human T-cell line which contains a single integrated copy of HIV-1 DNA (68), was used in serial 5-fold dilutions ranging from 0.01 ng

to 1 ng, the equivalent of 1 to 100 cells. PCR reactions were carried out in 50 μ l, containing the appropriate DNA concentration, 200 μ M each deoxynucleoside triphosphate, PCR buffer (50 mM KCl, 1.75 mM MgCl₂, 100 μ g nuclease-free bovine serum albumin [BSA], 20 mM Tris [pH 8.4]), 1 μ M each primer, and 2.5 U *Taq* DNA polymerase (Pharmacia). Reactions were carried in a 48-well automated Perkin Elmer Cetus thermal cycler.

β -actin amplifications involved 1 cycle of denaturation (94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec), and 1 cycle of extension (72°C for 10 min). Amplifications using *env* or *gag-pol* primers were carried out with 1 cycle of denaturation (95°C for 10 min), 35 cycles of amplification (95°C for 1 min, 55°C for 1 min, and 72°C for 2 min), and 1 cycle of extension (72°C for 10 min). Negative controls using uninfected human DNA and reagent controls without DNA were included in every experiment.

Amplified products were analyzed by electrophoresis in agarose gels, transfer to Nytran membranes (Schleicher & Schuell, Keene, NH), and hybridization with double-stranded DNA probes, which were random labelled with α [³²P]dATP (Du Pont, Boston, MA) and specific for actin, *env* or *gag-pol* sequences. Hybridizations were performed for 16 hours with 10⁶ cpm of radiolabelled probe per ml of buffer, and washed for 1 hour (83). Hybridizations and washes were carried at 55°C for

β -actin, and at 60°C for both *env* and *gag-pol*. Membranes were exposed to Fuji medical X-ray film at -80°C.

Proviral load calculations were done by comparison of the results of PCR amplifications of serial dilutions of patient samples to the serial dilutions of the 8E5 DNA standard curve using densitometry (4, 155). Amplifications carried out with β -actin primers and serial dilutions from subject and 8E5 DNA served as internal controls to assure equivalent amounts of input DNA. Proviral load was calculated in DNA from purified CD4⁺ T cells and PBMC obtained from 18 HIV-infected mothers. We, and others, have determined that similar viral load results can be obtained when quantitation is performed using either DNA from separated CD4⁺ T lymphocytes or from total PBMC, corrected to CD4⁺ T cells based on flow cytometry analysis of blood T cell subsets (208).

HIV-1 p24 antigen assay. HIV-1 antigenemia was determined by measuring the level of p24 antigen in maternal plasma after acid dissociation of immune complexes using an ELISA assay (Coulter, Hialeah, FL).

Statistical analysis. Paired statistical analysis of the results were carried out using Sigma Stat software (Jandel Scientific, San Rafaelo, CA). Comparison of viral load in CD4⁺ T lymphocytes and total PBMC in treated versus untreated, transmitting versus non-transmitting mothers, was performed using the Mann-Whitney rank sum test. Comparisons of CD4⁺ T cell counts, CD4 to CD8 ratios, and age within the study

populations were performed using Student t test. Fisher's exact was applied to compare each of the following: p24 antigenemia, mode of delivery, race, and CDC disease stage in the transmitting and non-transmitting groups, and in the treated and untreated groups. The rate of transmission in treated and untreated mothers was compared using a Fisher's exact test.

Results

Characteristics of study population. Clinical and demographic characteristics of the individual women enrolled in the study are shown in table 3.1. Forty-two HIV-1 infected pregnant women were enrolled prospectively. Women who had received antiretroviral therapy prior to their pregnancy were excluded. Most women (85.4%) gave birth by vaginal delivery. None of the infants were breast fed. The study group ranged in age from 14 to 37 years with a mean of 25.6 (\pm 5) years and were predominantly African American (77.5%). The clinical and immunological status of the study population at the time of delivery was highly variable. Ten of 42 women (23.8%) were symptomatic (CDC stage B or C). CD4⁺ T cell counts ranged from as low as 7 to as high as 1046 per microliter, with a mean CD4⁺ T cell count of 477 (\pm 294) cells per microliter.

Clinical, immunological and virological characteristics of mothers not treated with ZDV. Within the study population 23 women received no antiretroviral therapy during pregnancy.

Table 3.1. Clinical data for the cohort of HIV-1 mothers

Patient ^a	Age	Race ^b	Transmission status ^c	CDC stage	Mode of Delivery ^d	p24 Ag ^e	CD4 ⁺ T cells/ μ l	CD4:CD8 ratio
Untreated								
564	23	B	T	A1	VD	-	882	1.03
184	23	B	T	A1	VD	-	750	0.67
211	17	B	T	A1	VD	+	396	0.43
567	19	W	T	A1	VD	-	550	0.54
051	27	B	T	C3	VD	+	296	0.28
250	29	B	T	C3	VD	+	39	0.07
1400	31	H	T	A2	VD	+	68	0.07
402	30	B	T	C3	CS	+	7	0.03
559	32	B	T	C3	CS	+	133	0.12
313	26	B	T	C3	VD	+	102	0.12
179	29	B	T	C3	VD	-	38	0.01
566	23	B	NT	A1	VD	-	995	1.33
369	17	B	NT	A1	VD	-	590	0.57
1147	23	B	NT	A1	VD	-	824	0.80
570	25	B	NT	A1	VD	+	370	1.00
590	37	B	NT	A1	VD	-	539	1.53
541	23	B	NT	A3	VD	-	175	0.79
395	27	W	NT	A2	VD	-	586	0.40
319	26	B	NT	A1	VD	-	808	0.70
1314	14	B	NT	A1	VD	-	925	0.49
503	NA	NA	NT	A1	NA	NA	650	NA
352	24	B	NT	A1	VD	-	588	0.65
1005	28	B	NT	A2	VD	-	305	0.36
ZDV Treated								
1322	27	W	T	A1	CS	-	678	1.22
1211	29	NA	T	B2	VD	-	105	0.17
1427	18	W	NT	A1	VD	-	1046	0.83
1412	23	B	NT	A1	VD	+	800	NA
1222	28	B	NT	A1	VD	-	612	0.81
1384	18	B	NT	A1	VD	-	421	0.77
1391	18	B	NT	A1	VD	-	432	0.33
1260	28	B	NT	A1	VD	-	846	NA
1388	26	B	NT	A2	VD	-	357	0.47
1248	29	H	NT	A1	VD	-	671	NA
1089	27	B	NT	A1	CS	-	806	0.64
1149	37	B	NT	A1	CS	-	519	NA
1462	24	B	NT	A1	VD	+	430	NA
1401	28	W	NT	A1	VD	-	466	0.84
1310	21	W	NT	B2	VD	+	357	NA
1161	27	B	NT	A1	VD	-	501	0.45
1360	34	B	NT	A2	VD	-	223	0.17
1107	19	B	NT	B3	VD	+	123	0.12
1116	28	W	NT	B3	CS	+	47	0.12

Table 3.1 continued...

^a designated by numbers

^b B, African-American; H, Hispanic; C, Caucasian

^c T, transmitting; NT, non-transmitting

^d CS, Caesarean section; VD, vaginal delivery

^e +, detected; VD, vaginal delivery

NA, not available

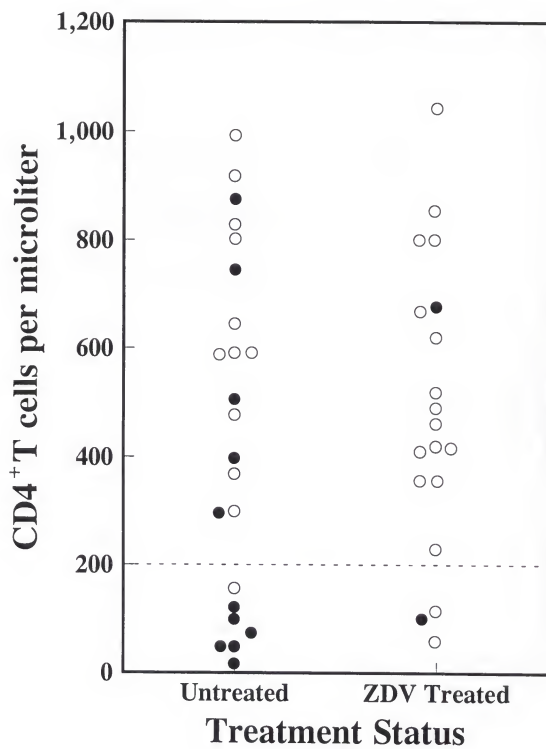
Neither were their newborns treated with ZDV. The untreated population was predominantly African American (86.4%) with an average age of 25 (\pm 5) years at the time of delivery (Table 3.2). Among the untreated mothers, 11 (48%) transmitted HIV-1 to their children while 12 mothers did not transmit the virus. Transmission in this cohort was higher than the 25% to 30% that is usually observed in our geographic region (39). A likely explanation for the transmission rate in our cohort was that universal HIV-1 screening of pregnant women was not in place at our study sites when enrollment began. Consequently, women with more symptomatic HIV disease and lower CD4⁺ T cell counts, who were at greater risk for transmission, were more likely to be identified during pregnancy and, therefore, were overrepresented in our untreated group. Because of this potential bias, we compared the rate of transmission between ZDV treated and untreated women with CD4⁺ T cell counts of greater than 200 cells per microliter. These women were more similar to the population of mothers enrolled in the ACTG protocol 076 (36). As shown in figure 3.1, the rate of transmission in women with CD4⁺ T cell counts greater than 200 cells per microliter in the untreated group was 31.3%, compared to 6.3% in the ZDV treated group ($p=0.083$). Overall, only 2 mothers (10.5%) in the ZDV treated group had infected infants, which was significantly different from transmission by untreated mothers ($p=0.017$). The results indicate that ZDV

Table 3.2. Clinical and virological characteristics of untreated mothers^a

Transmission status	Number	Age in years	Race ^b	CD4 ⁺ T cells/ μ l	CD4:CD8 ratio	p24 Ag ^c	HIV-1 copies/ 10^6 CD4 ⁺ T cells
Transmitters	11	26 (\pm 4.9)	82%	296 (\pm 309)	0.3 (\pm 0.3)	64%	2063 (\pm 4901)
Non-transmitters	12	24 (\pm 6)	91%	613 (\pm 249)	0.8 (\pm 0.4)	9%	67 (\pm 97)
p value		0.5	0.6	0.01	0.005	0.01	0.003

^a Results are expressed as mean (\pm standard deviation)^b Percent African-American^c Percent positive

Figure 3.1. CD4⁺ T cell counts in HIV-infected pregnant women. The number of CD4⁺ T cells per microliter of blood is shown on the y axis. Untreated mothers received no ZDV therapy during their pregnancy. The treated mothers and their infants received ZDV according to ACTG protocol 076. Open circles indicate mothers who did not transmit HIV-1 to their infants while the closed circles represent mothers who gave birth to infected children.



therapy administered to mothers and neonates in our cohort was effective in reducing the rate of transmission by 78%.

Mothers who transmitted HIV-1 did not differ significantly from the non-transmitting mothers with respect to mode of delivery, age (26 ± 4.9 compared to 24 ± 6 years) ($p=0.5$), or race ($p=0.6$) (Table 3.2). However, significant differences in both immunological and virological parameters between transmitting and non-transmitting mothers were identified. Mothers whose infants became infected had fewer CD4⁺ T cells, lower CD4 to CD8 ratios, and more advanced HIV disease than mothers whose infants were not infected. Six of 11 transmitting women (54.5%) were CDC stage C3. The group of 11 transmitting women had an average CD4⁺ T cell count of 296 (± 309) cells per microliter and a mean CD4 to CD8 ratio of 0.3 (± 0.3). In contrast, the 12 non-transmitting mothers were asymptomatic (CDC stage A1 to A3) with mean CD4⁺ T cell counts of 613 (± 249) cells per microliter, which was significantly greater than the CD4⁺ T lymphocyte count in the untreated transmitting group ($p=0.01$). Mean CD4 to CD8 ratio, 0.8 (± 0.4), in the non-transmitting group was also significantly greater than in the transmitting group of women ($p=0.005$).

Within the group of non-treated women, plasma virus was detected in 63.6% of transmitting mothers, but only in 9.1% of non-transmitting mothers ($p=0.01$). In addition, the differences existed between non-transmitting and transmitting mothers in mean numbers of HIV-1 DNA copies per 10^6 CD4⁺ T

cells was 67 (\pm 97) and 2063 (\pm 4901) respectively, which was significant ($p=0.003$).

Clinical, immunologic, and virologic parameters of ZDV-treated infected women. Nineteen HIV-1 infected women and their neonates received ZDV according to the ACTG protocol 076. To rule out the possibility that factors other than ZDV could account for reduced transmission among the women in our group, paired analysis of multiple parameters were examined between the untreated and treated groups of women (Table 3.3) (55, 63, 135). ZDV-treated women were the same age as the untreated group and there were similar numbers of symptomatic women, 21% versus 26% respectively ($p=0.7$). Although the ZDV-treated group had more deliveries by Caesarean section (21% versus 9% in untreated subjects), and included fewer African Americans (67% versus 86%), the differences between the treated and untreated groups in our study did not reach statistical significance.

When immunological parameters were examined, the ZDV-treated group was virtually identical to the untreated group of women in CD4⁺ T cell numbers, 462 (\pm 317) and 497 (\pm 270) respectively ($p=0.7$), and CD4 to CD8 ratio ($p=0.9$) (Table 3.3). Virological parameters were also similar between the ZDV-treated and untreated groups of women. Five of 19 ZDV-treated women (26%) had detectable p24 antigen, which was not significantly different from the untreated group in which antigenemia was detected in 8 of 22 women (36%) ($p=0.2$).

Table 3.3. Clinical and virological characteristics of ZDV-treated versus untreated mothers^a

Treatment status	Number	Age in years	Race ^b	CD4 ⁺ T cells/ μ l	CD4:CD8 ratio	p24 Ag ^c	Transmission	HIV-1 copies/ 10 ⁵ CD4 ⁺ T cells
Treated	19	26 (\pm 5)	67%	462 (\pm 317)	0.5 (\pm 0.4)	26%	10.5%	1022 (\pm 3458)
Untreated	23	25 (\pm 5)	86%	497 (\pm 270)	0.5 (\pm 0.4)	36%	48%	1172 (\pm 4568)
p value		0.7	0.3	0.7	0.9	0.2	0.02	0.3

^a Results are expressed as mean (\pm standard deviation)

^b Percent African-American

^c Percent positive

CD4⁺ T lymphocytes and HIV-1 infection in individual untreated and ZDV-treated women. Numbers of infected maternal peripheral blood lymphocytes within both the ZDV-treated and untreated groups ranged from 13 to 20,000 per 10⁶ CD4⁺ T cells (Fig. 3.2). Among the total of 42 women in our population, 29 did not transmit the virus. When the 17 ZDV-treated and the 12 untreated women who did not transmit the virus to their children were analyzed, no significant differences in clinical or virological parameters were detected between the 2 groups. In addition, the mean HIV-1 DNA copy number per 10⁶ CD4⁺ T cells in the treated women, 1022 (\pm 3458), was similar to the untreated group, 1172 (\pm 4568) ($p=0.3$).

Among untreated mothers with fewer than 100 copies of HIV-1 per 10⁶ CD4⁺ T cells, 3 of 13 (23%) transmitted. In the ZDV-treated group, two of 15 mothers (13.3%) with fewer than 100 copies per 10⁶ CD4⁺ T cells transmitted HIV-1 to their infants. The difference in transmission between the ZDV-treated and untreated mothers with this low level of cell associated virus in CD4⁺ T cells was not significant ($p=0.6$).

A difference in maternal transmission became apparent among women who had greater than 100 copies of HIV-1 per 10⁶ CD4⁺ T cells (Fig. 3.2). Among 10 untreated mothers with > 100 HIV-1 copies (range 150 to 16,665) per 10⁶ CD4⁺ T cells, eight (80%) transmitted. In contrast, none of the 4 ZDV-treated mothers with > 100 (range 270 to 20,000) copies of HIV-1 per million CD4⁺ T cells transmitted the virus. There was no

Figure 3.2. HIV-1 copies in peripheral CD4⁺ T cells in women who were untreated or ZDV-treated. Open circles indicate women who did not transmit HIV-1 to their children. Closed circles represent women whose infants became HIV-1 infected. Among the untreated group, 3 mothers (184, 211 and 564), who had <10² HIV-1 copies per million CD4⁺ T cells, transmitted; 2 mothers (352 and 1005) with >10² HIV-1 copies per million CD4⁺ T cells did not transmit. Among the ZDV-treated group, 4 mothers (1360, 1161, 1107 and 1116) with >10² HIV-1 copies did not transmit to their infants.

significant difference in mean levels of HIV-1 DNA copies in CD4⁺ T cells between the 2 groups (2311 \pm 5096 versus 5450 \pm 9711 in untreated and treated, respectively; $p>0.1$). Yet, the difference in transmission between untreated and ZDV-treated mothers and neonates was significant ($p=0.015$).

Discussion

In the absence of antiviral therapy for HIV-1 infected mothers and their infants, a significant relationship between maternal immunological and virological parameters and risk of HIV-1 infection for the infant was detected in our study. An increased likelihood for transmission within our cohort of untreated HIV-1 infected women was inversely related to maternal CD4⁺ T cell counts and CD4 to CD8 ratios, and directly associated with levels of HIV-1 DNA copies found in CD4⁺ T cells in maternal peripheral blood.

A positive relationship between pediatric infection and levels of maternal virus was found in other studies based on evaluation of HIV-1 either in plasma, by p24 or RNA PCR, or in cells, primarily by culture (64, 65, 97, 155, 202). We relied on 2 measurements of HIV-1 infection in the mothers, specifically p24 plasma antigenemia and HIV-1 DNA copies within PBMC. Most untreated women in our population were enrolled prior to widespread use of plasma RNA levels as a clinical test, so for consistency p24 antigen was evaluated for all women in our study population. Most likely the number

of mothers with detectable plasma viremia was underestimated in our study because measurements of plasma viremia by p24 antigen capture are less sensitive than quantitative viral RNA assays (25). Nonetheless, there was general concordance between detectable plasma viremia and transmission among the mothers in our study. Amplification of HIV-1 DNA was sensitive enough in our assays to detect one copy of HIV-1 in DNA from 150,000 cells. The level of HIV-1 provirus per million peripheral CD4⁺ T lymphocytes ranged from 13 to 20,000 among the women in our population, which is comparable to levels of HIV-1 infection in adults determined by similar methodology (34, 139, 208).

Our study population was comprised of non-randomized subjects and included untreated mothers who were equally divided between transmitters and non-transmitters. However, ZDV treatment administered according to ACTG protocol 076 to the mothers and neonates in our population, resulted in about 10% pediatric infection, which is similar to the results from the original trial (36). The impact of ZDV treatment on maternal viremia or cell-associated virus was not evaluated as part of the original ACTG protocol 076. In our study, ZDV treatment significantly reduced HIV-1 infection among the infants, but the effect was independent of maternal immunological or virological parameters. In fact, the two ZDV-treated women who transmitted in our population had fewer than 100 HIV-1 copies per million CD4⁺ T cells and undetectable

plasma viremia. ZDV-resistant virus was detected in one treated mother, which could account for transmission in that case, and suggests that even relatively low levels of resistant virus increases the risk for pediatric infection (69).

Transmission also occurred in the absence of ZDV treatment among 3 mothers with fewer than 100 HIV-1 copies per million CD4⁺ T cells. No other confounding clinical factors that would account for transmission were readily discernible among these mothers or their infants. Viral phenotype was not evaluated prospectively as part of these particular studies. However, viral characteristics, such as macrophage tropism or syncytium formation in culture, have been implicated as factors in maternal transmission (92, 111, 140, 150, 166, 175, 196).

Based on our results, ZDV appears to exert its greatest effect on reducing maternal transmission among women with high proviral load. These results raise a question as to how ZDV administered to mother and neonate reduces HIV-1 infection. If the effect is not on levels of virus in the mother, then other mechanisms must account for the success of ZDV therapy. For example, ZDV may reduce infectivity of maternal virus by selection of viruses with increasing resistance to the drug. Multiple amino acid substitutions in reverse transcriptase (RT) are required for high level ZDV resistance of the virus. Intermediate amino acid changes can result in reduced drug sensitivity and altered virus viability (101). Alternatively,

ZDV may have an impact on the target cells in the neonate that are susceptible to infection. The effect of ZDV on nucleic acid elongation is not restricted to DNA synthesis by HIV-1 reverse transcriptase (72). Replication of host cell mitochondrial and chromosomal DNA is also impacted by nucleoside analogues such as ZDV. ZDV produces a transient suppressive effect on neonatal hematopoiesis (36, 177). It has also been shown to reduce the proliferative response of lymphocytes *in vitro* (88). A clearer understanding of the factors which enable ZDV to reduce HIV-1 infection in neonates is essential to develop more effective therapeutic strategies that will essentially eliminate pediatric HIV-1 infection by maternal transmission.

CHAPTER 4
PEDIATRIC HIV-1 INFECTION OF MONOCYTES
AND CD4⁺ T LYMPHOCYTES

Introduction

Mother-to-child transmission of HIV-1 accounts for most infections in the pediatric population. Different than adult individuals infected with the virus, vertically infected children usually present a more rapid progression to disease. Multiple factors can have an impact on the disease outcome in children including timing of maternal transmission of HIV-1 to the infant, immaturity of the neonatal immune system (109), and increased susceptibility of neonatal monocytes-macrophages to HIV-1 infection (185).

Children who have virus detected in their peripheral blood by PCR (polymerase chain reaction) or culture at birth are defined as being infected in utero, and seem to develop symptoms faster than children who acquire HIV later during gestation (PCR or culture negative at birth) (48). Also, because young children have significantly fewer memory (CD45RO) CD4⁺ T cells than adults (22% in normal children in the first 2 years of life versus 63% in adults) (94), immunological functions such as T cell proliferation in response to antigens and production of antigen-specific IL-2

and γ -interferon, could be more depressed in infected children than in adults.

The role of monocytes and macrophages in the immunopathogenesis of HIV infection is not completely clear. In contrast to the decline in $CD4^+$ T lymphocytes that occurs during HIV infection, changes in monocyte-macrophage number are minimal even in late stage disease (126, 127). Monocyte-macrophage function in HIV-1 infected individuals has been described to be impaired by some, but not by other authors. Reports concerning oxidative burst, candidacidal activity, chemotaxis and phagocytic function of monocytes-macrophages are controversial (18, 26, 67, 138, 146). Possible reasons for the contradictory reports could be that (1) HIV-1-infected peripheral blood monocytes have been identified in only a small proportion of infected individuals (169), and (2) infected cells of this lineage are not necessarily killed by the virus (32, 126, 170, 172). It is thought that the infected cells may function as a reservoir of virus in different organs, particularly the brain and lung. Incidence of HIV-1-related central nervous system (CNS) disease in infants and children is greater than in adults, and it is estimated to affect 30-40% of symptomatic children (21, 23). HIV-infected monocytes-macrophages may also be involved in the spread of virus to $CD4^+$ T cells, and infection of placental macrophages could play a role in the vertical transmission of HIV-1 (118, 175).

The goals for this part of our study were (1) to define the role of blood monocytes in the immunopathogenesis of HIV-1 infection in children, and in the vertical transmission of the virus, and (2) to compare progression of disease in a pediatric population, with diverse outcome during the first year of life. Children 0-13 years of age were studied longitudinally or cross-sectionally. First, because infection of blood monocytes is a controversial subject, it was very important to characterize extensively the presence of virus in these cells. A technique of monocyte isolation was developed for this purpose which yields monocytes that can be 98-99% depleted of T lymphocytes (4). The great advantage of this technique over others described is the ability to verify the presence of residual T lymphocytes among the selected monocyte population, essential to avoid misleading results (4). Second, characteristics of virus replication in children who were infected early versus late during pregnancy, and in slow and rapid progressors, were compared using a PCR based assay. To understand with more details the behavior of the virus in pediatric HIV, infection of naive (CD45RA) and memory (CD45RO) subsets of CD4⁺ T cells was then analyzed.

Materials and Methods

Patients. HIV-1 infected individuals were enrolled in this study at the Pediatric Immunology Clinic at the University of Florida, Gainesville, FL, from June of 1989

through April of 1996. Signed informed consent was obtained from all mothers.

Thirteen HIV-1 vertically infected children were followed prospectively from birth, with a total of 92 blood samples analyzed from these individuals (Table 4.1). Five of these children were HIV PCR positive at birth (determined within 24 hours of birth), while 8 children did not have virus detected in PBMC (peripheral blood mononuclear cells) by PCR at that time. None of these infants were breast-fed. A group of 3 HIV-1 infected children 2 to 12 years old was also followed prospectively, with a total of 20 blood samples analyzed (Table 4.2). One of these children (MIST) was infected by contaminated blood product at birth, while the 2 other were vertically infected by their HIV-1 positive mothers. Status of infection was not determined in these 2 children at the time of birth. Cross-sectional analysis included blood samples from 9 HIV-1 children 1 to 13 years of age (Tables 4.1 and 4.2). One child (NIHI) was infected by contaminated blood product at birth, while the other 8 acquired HIV-1 from their infected mothers. Clinical information regarding PCR results at birth was not available for these children. None of the vertically infected children in this study was treated with zidovudine (ZDV) according to the ACTG 076 protocol, to prevent maternal transmission of HIV-1.

Virological and clinical data collected from these children included gestational age at birth, race, CD4 and CD8

Table 4.1. Characteristics of HIV-1 infected children followed longitudinally from birth, classified according to pattern of disease progression^a

Patient ^b	Age in months	CD4 ⁺ T cells/ μ l	CD4:CD8 ratio	CDC stage	HIV-1 copies/ 10^6 CD4 ⁺ T cells	
Slow progressors						
MEST	131	4.5	3969	1.8	N1	29
	160	7.0	3969	1.8	A1	82
	189	9.0	3969	1.8	A1	122
	299	14.4	1889	0.9	A1	188
	400	28.0	972	0.3	B2	125
	461	31.0	972	0.3	B2	400
	513	37.0	544	0.3	B2	235
	1281	61.0	440	0.3	B3	125
	1481	69.0	317	0.3	B3	89
	1555	72.0	388	0.2	B3	200
KAAH	49	0.3	2312	3.0	E	ND
	83	3.0	2312	3.0	N1	167
	113	8.3	1155	2.0	A2	104
	166	13.3	1155	2.0	A2	375
	280	28.0	1466	2.3	A2	87
	383	34.0	1499	2.3	B2	43
	539	46.0	1268	2.2	B2	53
	1082	58.0	647	1.8	B2	48
	1290	69.0	682	1.4	B2	54
	1399	73.0	630	1.4	B2	52
	1428	74.0	630	1.4	B2	20
	1503	77.0	648	1.0	B2	20
DEWA	1056	8.5	2003	1.0	N1	25000
	1101	11.0	1490	0.6	A1	30000
	1133	12.8	2145	0.7	A1	8000
	1164	14.3	767	0.4	A2	1180
	1221	17.0	677	0.3	A2	670
	1297	21.5	1214	0.5	A2	1000
	1362	25.0	1214	0.5	A2	300
	1443	28.0	539	0.4	A2	40
	1532	30.0	1130	0.4	B2	100
DIKA	1069	9.5	1633	1.6	A1	10000
	1180	15.0	1455	1.0	A1	1480
JOSH	1242	8.3	4236	1.7	A1	100
	1256	9.0	3632	1.9	A1	37
	1414	15.5	1720	1.1	A1	334
	1470	18.0	1976	0.7	A1	33
	1536	20.0	2755	0.7	A1	1400
TEWI ^c	1547	18.0	1085	1.5	N1	160

Table 4.1 continued...

Patient ^b	Age in months	CD4 ⁺ T cells/ μ l	CD4:CD8 ratio	CDC stage	HIV-1 copies/ 10^6 CD4 ⁺ T cells
Rapid progressors					
JOFO	1492	0.9	1789	1.5	N1 2325
	1516	1.8	1171	1.2	N1 1200
	1542	2.8	977	0.4	N2 12500
SAFR	1016	8.3	502	0.4	C3 8000
	1039	9.5	498	0.3	C3 10000
	1068	11.8	484	0.3	C3 8000
	1132	15.0	412	0.4	C3 3350
	1197	17.8	277	0.4	C3 1880
	1232	20.5	128	0.2	C3 500
	1296	24.0	126	0.3	C3 1430
	1356	27.0	126	0.3	C3 1143
	1440	30.0	126	0.3	C3 571
	1530	33.0	45	0.05	C3 700
JERO	576	1.0	1176	0.6	C2 10000
	1029	5.0	722	0.7	C2 30000
	1076	8.0	862	0.8	C2 25000
	1199	14.0	588	0.7	C2 1880
	1284	19.3	454	1.1	C2 300
	1426	25.0	664	0.7	C3 273
	1448	26.0	664	0.7	C3 91
	1537	29.0	1000	0.85	C3 1200
JALA	1004	1.5	3045	1.4	N1 1150
	1007	2.0	3045	1.4	A1 30000
	1038	4.5	1784	1.9	B1 50000
	1084	7.0	1784	1.9	B1 2000
	1120	9.0	1784	1.9	C1 425
	1201	12.5	902	0.7	C2 270
	1246	16.0	619	1.0	C2 10
	1268	17.0	619	1.0	C2 13
	1342	21.0	143	0.4	C3 5
	1415	23.0	96	0.2	C3 5
SHFI	413	1.0	2546	2.4	A1 10000
	458	3.8	1603	1.1	A1 2000
	588	17.0	62	0.1	C3 1000
	1225	30.5	385	0.2	C3 60
	1279	34.0	601	0.6	C3 100
	1346	36.0	485	0.06	C3 167
	1376	37.0	485	0.06	C3 167
	1447	41.0	238	0.4	C3 2667
	1539	44.0	428	0.4	C3 100
SHJO	480	0.03	1835	1.6	N1 95
	494	1.3	2394	1.6	A1 10000
	530	5.8	1597	1.9	A1 5000
	1162	22.0	1134	0.6	A1 140
	1271	28.0	924	0.9	A1 29

Table 4.1 continued...

Patient ^b	Age in months	CD4 ⁺ T cells/ μ l	CD4:CD8 ratio	CDC stage	HIV-1 copies/ 10^6 CD4 ⁺ T cells
Rapid progressors					
1331	32.0	857	0.9	A1	29
1471	36.0	704	0.9	A2	29
MEBE ^c 1308	12.0	48	0.9	C3	2857
Indetermined					
NIBA 1474	3.0	4111	0.7	N1	130
1498	3.8	4111	0.7	N1	130
1508	4.0	2083	0.8	N1	194
1518	4.3	2359	0.7	N1	2000
REGR 1259	0.03	2059	1.5	E	ND
1278	1.0	3338	2.4	N1	1000
1443	7.5	1665	1.1	A1	20

^a samples from two patients who were class E at the time, were not included in the statistical analysis

^b designated by initials

^c patients analyzed at one time point only, not included in the statistical analysis

ND, not detected

Table 4.2. Characteristics of HIV-1 infected children 2-13 years of age

Patient ^a	Age in years		CD4 ⁺ T cells/ μ l	CD4:CD8 ratio	CDC stage	HIV-1 copies/ 10 ⁶ CD4 ⁺ T cells
MIST ^b	22	5.8	1059	0.4	A1	182
	246	7.9	64	0.4	A1	250
	407	8.9	591	0.2	A2	615
	535	9.8	795	0.3	A2	235
	1072	10.7	884	0.3	A2	300
	1229	11.4	728	0.2	A2	90
CHST	122	2.7	1329	0.9	B1	1540
	196	3.0	1012	0.8	B1	1548
	387	4.5	1131	0.7	B1	1290
	527	5.3	812	1.1	B1	1250
	1042	6.3	938	0.7	B1	1935
	1224	7.1	620	0.9	B1	150
	1335	7.6	765	1.0	B1	1360
	1431	7.8	800	0.6	B1	100
JOST	158	2.4	933	0.6	B1	148
	307	3.0	933	0.6	B1	222
	399	4.2	578	0.2	B2	182
	556	5.3	558	0.3	B2	223
	1282	7.0	246	0.2	B3	200
	1438	7.5	224	0.2	B3	20
NIHI ^b	1551	12.0	638	0.9	B1	10
MEMO	1112	8.8	913	1.2	A1	200
ALGA	504	7.7	11	0.03	C3	220
JOLE	432	5.1	386	0.5	C2	150
	1254	7.6	210	0.3	C2	500
JASM	1169	7.7	270	0.6	C3	100
	1194	7.8	357	0.8	C3	300

^a designated by initials^b patients infected by contaminated blood product at birth

T lymphocyte counts, p24 antigenemia, percent monocytes, percent HLA-DR⁺ T lymphocytes, use of antiretroviral therapy, and disease stage according to the 1994 classification system by the Centers for Disease Control (CDC), as follows: immunologic categories 1 (no evidence of suppression); 2 (evidence of moderate suppression); and 3 (severe suppression). Clinical categories E (HIV status not confirmed); N (no signs/symptoms); A (mild signs/symptoms); B (moderate signs/symptoms); and C (severe signs/symptoms) (31). Children <18 months of age were defined as infected if they had virus detected by PCR on at least two occasions. Children >18 months of age were defined as infected if they had a positive PCR and/or ELISA, and confirmed by Western blotting.

Cross-sectional analysis of blood samples from 16 HIV-1 mothers was also performed. Among these women there were 11 mothers who did not receive ZDV to prevent vertical transmission of HIV-1, 7 of whom transmitted the virus to their children. The other 5 women received ZDV according to the ACTG 076 protocol. There were no transmitting mothers in this group (Table 4.3).

Virologic and clinical data collected from the adult patients included age, race, CD4 and CD8 T lymphocyte counts, p24 antigenemia, percent monocytes, disease stage according to the CDC classification (194), status of transmission to the child, and use of antiretroviral therapy to prevent vertical transmission of HIV-1.

Table 4.3. Detection of $V_{\beta}8$ and HIV-1 sequences in DNA from $CD14^+$ monocytes and $CD4^+$ T lymphocytes of infected mothers

Patient ^a		V _β 8/10 ⁴ cells		HIV-1/10 ⁴ cells		% CD14 ⁺ cells ^b
		CD4 ⁺ T	CD14 ⁺	CD4 ⁺ T	CD14 ⁺	
Transmitting						
CODI	1015	50	20	2	<1	7.0
	1106	6	3	0.2	<1 ^c	7.0
TEBU	1014	50	2	5	1	11.5
MARA	578	ND	ND	4	<1	5.0
LOWA	1102	ND	ND	100	<1	NA
CABR	1096	8	2	0.2	<1 ^c	14.5
ROMO	1111	ND	ND	70	<1	9.0
HAST	433	4	1	4	0.6	12.0
Non-transmitting						
ELHU	1033	20	6	0.2	<1	7.0
DERU	1000	ND	ND	0.4	<1	NA
CHWA	1052	ND	ND	1	<1	3.0
LAOW	1147	ND	ND	0.1	<1 ^d	NA
076 non-transmitting						
PAEL	1152	12	<1	0.7	<1 ^e	NA
LIWA	1089	ND	ND	0.3	<1	7.6

Table 4.3 continued...

Patient ^a	V _p 8/10 ⁴ cells		HIV-1/10 ⁴ cells		% CD14 ⁺ cells ^b
	CD4 ⁺ T	CD14 ⁺	CD4 ⁺ T	CD14 ⁺	
076 non-transmitting					
JAWI 1116	ND	ND	200	<1	4.0
DICO 1215	ND	ND	0.5	<1	6.6
DERO 1222	20	5	0.2	<1 ^c	3.0

^a designated by initials

^b percent of total PBMC according to flow cytometry analysis

^c less than 1 copy of HIV-1 detected in 5 X 10⁴ cells

^d less than 1 copy of HIV-1 detected in 7.5 X 10⁴ cells

^e less than 1 copy of HIV-1 detected in 1.5 X 10⁴ cells

ND, not done; bold letters, samples also amplified with M667-AA55 primers

Cell lines. Cell lines used for control experiments were Jurkat, a human T-cell line which has rearranged $V_{\beta}8-J_{\beta}1.2$ T cell receptor (TCR) (203), and 8E5, a human T-cell line which contains a single integrated copy of HIV-1 DNA in each cell (68). The cell lines were obtained from the NIAID AIDS Research and Reference Program.

CD14⁺ monocytes, CD4⁺ T lymphocytes and CD4⁺ CD45RA/CD45RO selection technique. Three to five milliliters of peripheral blood from children <2 years of age to up to 10 ml of blood from older children and adults, were collected in acid citrate dextran (ACD) tubes. All samples were processed within 24 hours after collection, as previously described (4). Briefly, plasma was separated from total blood and PBMC were collected after Ficoll-Hypaque density centrifugation (Histopaque-1007; Sigma Diagnostics, St. Louis, MO). CD14⁺ monocytes were selected from total PBMC after incubation of the cells with a mouse monoclonal antibody (MAb) anti-CD14, MY4 (125 $\mu\text{g/ml}$) (Coulter Immunology, Hialeah, FL), followed by incubation with immunomagnetic beads coated with sheep anti-mouse immunoglobulin G (IgG) (Dynabeads M450; Dynal, Oslo, Norway). Monocytes with beads were separated from the CD14-negative fraction using an MPC-1 Dynal magnet. CD4⁺ T lymphocytes were selected from the CD14-depleted cell fraction after incubation with a MAb anti-CD4, T4 (500 $\mu\text{g/ml}$) (Coulter Immunology) and immunomagnetic beads.

After depletion of CD14⁺ monocytes the CD4⁺ T lymphocytes were selected from the remaining PBMC using a MAb anti-CD4 linked to a detachable magnetic bead (DETACHaBEAD, Dynal), as previously described (181). After beads were detached from the CD4⁺ T lymphocytes, CD45RO cells were selected by incubation with UCHL1 mouse MAb (277 µg/ml) (Dako Corporation, Carpinteria, CA), and magnetic microspheres. CD45RA cell fraction was selected from the CD45RO-depleted CD4⁺ T cells, using MAb Leu-18 (25 µg/ml) (Becton Dickinson Immunocytometry Systems, San Jose, CA) and immunoaffinity beads.

Cell lysis and DNA extraction. Cells were resuspended in 1 ml lysis buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 2% sodium dodecyl sulfate [SDS], 100 µg proteinase K per ml) and incubated overnight at 37°C. DNA was extracted using the G NOME DNA isolation kit (Bio 101, Inc., La Jolla, CA), according to the manufacturer. DNA concentration was calculated by spectrophotometric reading using a Beckman DU 640 instrument.

Alternatively, cells were lysed in K buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM MgCl₂, 0.5% Tween 20 and 100 µg of proteinase K per milliliter of buffer), at a final concentration of 50 ng of DNA (5,000 cells) per microliter of K buffer.

PCR amplification. Primers used for amplification of HIV-1 were directed to the env region, forward LV15 (5'-GCCACACATGCCTGTGTACCCACA-3') and reverse 194G (5'-

CTTCTCCAATTGTCCCTCATA-3'), located at nucleotides 6464 to 6487 and 7693 to 7713, and to *gag-pol* sequences, forward *Gag1* (5'-GACCAGCAGCTACACTAGAAGA-3') and reverse *Pol2* (5'-TGCGGGATGTGGTATTC-3'), located at nucleotides 1802 to 1823 and 2863 to 2879, in the HIV-1_{LAI} genome as previously described (5). A third set of primers was used to amplify long terminal repeat (LTR) sequences of the virus, M667, forward (5'-GGCTAACTAGGGAACCCACTG-3'), corresponding to nucleotide positions 496 to 516, and AA55, reverse (5'-CTGCTAGGAATTTCCCACTGAC-3'), corresponding to nucleotides 612 to 635 (214). Primers specific for the V_β8 family of TCR were forward V_β8 (5'-GTTCCGATAGATGATTCAGGGATGCC-3') and reverse J_β1.2 (5'-TACAACGGTTAACCTGGT-3'), as previously described, yielding a 180 bp fragment (4). Amplifications of V_β8 sequences were performed to evaluate the level of enrichment of CD14⁺ monocytes, in relation to the presence of residual T cells. Oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer in the DNA synthesis core facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. β -Actin sequences were amplified with a forward primer (5'-GAAACTACCTTCAACTCCATCATG-3') and a reverse primer (5'-CTAGAAGCATTGCGGTGGAC-3') (Clontech, Palo Alto, CA). Amplified products were 350 bp. β -Actin amplifications were always performed to assure analysis of similar amounts of input DNA among individuals, and also

within the same individual when several PBMC subsets were evaluated for the presence of HIV-1.

All amplifications were performed in a total volume of 50 μ l. Concentrations of patient DNA used for amplifications were 0.1 and 1 ng for β -actin, 50 and 100 ng for $V_{\beta}8$, 50 to 1,000 ng for *env* and *gag-pol*, and 25, 50 and 100 ng for LTR (strong-stop DNA). DNA from Jurkat and 8E5 cell lines was used in 5-fold serial dilutions at concentrations that ranged from 0.01 to 1 ng (1 to 100 cells). Reactions also contained 200 μ M of each deoxynucleoside triphosphate (dTP), PCR buffer (50 mM KCl, 1.75 mM $MgCl_2$, 100 μ g of bovine serum albumin [BSA], 20 mM Tris [pH 8.4]), 1 μ M each primer, and 2.5 U of Taq DNA polymerase (Pharmacia). For amplification of LTR sequences, 5 mM $MgCl_2$ were used instead (214). Amplifications were performed in a 48-well automated Perkin-Elmer Cetus thermal cycler.

β -Actin was amplified by 1 cycle of denaturation (94°C for 5 min), 25 cycles of amplification (94°C for 30s, 60°C for 30s, and 72°C for 30s), and 1 cycle of extension (72°C for 10 min). $V_{\beta}8$ -J β 1.2 amplifications were performed with 1 cycle of denaturation (94°C for 5 min), 35 cycles of amplification (denaturation for 1 min at 94°C, annealing for 1 min at 45°C, and extension for 2 min at 72°C), and 1 cycle of extension (72°C for 10 min). Amplifications using the *env* and *gag-pol* primers were done with 1 cycle of denaturation (95°C for 10 min), 35 cycles of amplification (95°C for 1 min, 55°C for 1

min, and 72°C for 2 min), and 1 cycle of extension (72°C for 10 min). LTR (strong-stop) sequences were amplified by 1 cycle of denaturation (95°C for 10 min), 25 cycles of amplification (91°C for 1 min, and 65°C for 2 min), and 1 cycle of extension (72°C for 10 min) (214).

PCR products were electrophoresed in agarose gels and transferred to Nytran membranes (Schleicher & Schuell, Keene, NH). Double-stranded DNA probes specific for actin, *V_β8*, *gag-pol* and *env* sequences were random labelled with α [³²P]dATP (Du Pont, Boston, MA) to a specific activity of 1-3 X 10⁹ cpm/ μ g DNA, and hybridized to the appropriate products. Hybridizations were carried out for 16 hours with 10⁶ cpm of radiolabelled probe per ml of phosphate hybridization buffer (500 mM NaPO₄ [pH 7.2], 1 mM EDTA [pH 8.0], 7% SDS, 1% BSA), and washes were performed for 1 hour in 40 mM NaPO₄ with 0.1% SDS, at 55°C for β -actin, at 50°C for *V_β8*, and at 60°C for *gag-pol* and *env* analysis. M667-AA55 products (strong-stop DNA) were hybridized to an oligonucleotide probe, located at positions 561 to 582, radiolabelled with γ [³²P]ATP (Amersham Corporation, Arlington Heights, IL). Probe labelling was performed in a 25 μ l reaction containing 1:10 volume of 10 X kinase buffer (50 mM Tris-HCl [pH 8.2], 10 mM MgCl₂, and 5 mM dithiothreitol), 10 pmol oligonucleotide probe, γ [³²P]ATP (6,000 Ci/mmol), T4 polynucleotide kinase (10 U/ μ l). Membranes were placed in hybridization solution (6 X SSC, 10 mM EDTA [pH 7.5], 2 X Denhardt's solution, 100 μ g/ml salmon sperm DNA, and

0.5% SDS) for 16 hours at 55°C with 4 X 10⁶ cpm of radiolabelled probe per ml of buffer, and washed for 1 hour (20 X SSC, and 10% SDS), at 55°C. Hybridized filters were exposed to Fuji medical X-ray film at -80°C. Sensitivity of β -actin, *V_β8*, *gag-pol* and *env* primers was at the level of 1 to 5 cells. Level of detection of M667-AA55 primers was 50 cells.

Calculations of cell-associated viral load were performed by comparing results of patient DNA amplifications to amplification of an 8E5 DNA standard curve, which was included in every experiment. Intensity of hybridization was determined by densitometry (129).

Statistical analysis. Analysis were performed using a software program (Sigma Stat, Jandel Scientific, San Rafaelo, CA). Mann-Whitney rank sum test was used for viral load comparisons. t test was applied for comparisons of CD4⁺, and CD8⁺ T cell counts, CD4 to CD8 ratios, monocyte percentages and HLA-DR⁺ T lymphocyte percentages. CDC disease stage was compared by Fisher's exact test.

Results

Cell selection and enrichment of CD14⁺ monocytes. The initial step in analyzing infection by HIV-1 of pediatric PBMC was to determine which cell populations harbored the virus. Using immunoaffinity magnetic beads, CD14⁺ monocytes were selected from total PBMC, followed by selection of CD4⁺ T lymphocytes from the CD14-depleted cell fraction. According to

a technique previously described (4), we can select blood monocytes as much as 98 to 99% depleted of CD4⁺ T cells. This method is based on the detection of TCR rearrangements in the separated cells by PCR amplification of V_β8 sequences, a family of TCR detected in 3-26% of peripheral blood T lymphocytes (84, 148). Verification of monocyte enrichment was important when HIV-1 sequences were detected in these cells, to exclude the possibility that infected CD4⁺ T lymphocytes were responsible for viral amplification in monocytes. A total of 66 blood samples were analyzed (Tables 4.3, 4.4, and 4.5). Serial dilutions of patient DNA from both CD14⁺ monocytes and CD4⁺ T lymphocytes, ranging from 50 to 750 ng, were amplified with *gag-pol* (20 samples), *env* (34 samples), or with both primers (12 samples). Actin amplification performed in all samples demonstrated equal amounts of input DNA.

V_β8 sequences were amplified in 26 of the 66 samples. Monocytes ≥ 90% free of T cells were obtained in 13/26 cell separations. In 10 occasions (CODI [both samples], CABR, HAST, ELHU, DERO, MEST [31.0 mo], JERO [19.3 mo], JALA [2.0 mo] and JOLE [5.1 yo]), unsatisfactory monocyte selection was performed, yielding cells <80% enriched. Many of these samples were manipulated during initial optimization of the technique, and slight differences in the cell selection process could account for the inferior results. Fortunately, in most cases the quality of the selection technique did not affect interpretation of the assays (see below).

Table 4.4. Detection of $V_{\beta}8$ and HIV-1 sequences in DNA from $CD14^+$ monocytes and $CD4^+$ T lymphocytes of infected children followed longitudinally from birth

Patient ^a	Age in months	$V_{\beta}8/10^4$ cells		HIV-1/ 10^4 cells		% $CD14^+$ cells ^b
		$CD4^+$ T	$CD14^+$	$CD4^+$ T	$CD14^+$	
MEST	461 31.0	8	2	4	2	13.7
	1070 50.0	ND	ND	$\geq 250^c$	1	5.0
	1555 72.0	ND	ND	2	$<1^d$	5.0
DEWA	1056 8.5	20	1	250	<1	3.0
	1101 11.0	10	2	300	1	9.0
	1297 21.5	ND	ND	10	1	9.0
	1443 28.0	ND	ND	0.4	<1	7.0
	1532 30.0	ND	ND	1	$<1^c$	10.0
DIKA	1069 9.5	10	2	100	2	13.0
JOSH	1242 8.3	ND	ND	1	$<1^d$	3.0
	1414 15.5	ND	ND	3.3	<1	5.0
	1536 20.0	ND	ND	14	0.4	6.0
TEWI	1547 18.0	ND	ND	1.6	$<1^d$	16.0
JOFO	1516 1.8	ND	ND	12	$<1^c$	NA
SAFR	1016 8.3	80	5	80	2	10.0
	1039 9.5	50	4	100	2	3.0
	1068 11.8	40	10	80	4	4.0
	1232 20.5	ND	ND	5	<1	23.0
	1296 24.0	ND	ND	14.3	<1	6.0
	1440 30.0	ND	ND	5.7	$<1^d$	10.6
	1530 33.0	ND	ND	7	0.6	1.0
JERO	576 1.0	10	1	100	1	9.0
	1029 5.0	10	1	300	1	10.0
	1076 8.0	10	1	250	1	10.0
	1284 19.3	10	5	3	<1	8.0
	1537 29.0	ND	ND	12	0.4	6.0
JALA	1007 2.0	20	8	300	4	11.0
	1084 7.0	10	1	20	<1	5.0
	1268 17.0	ND	ND	0.13	$<1^d$	11.0
	1415 23.0	ND	ND	0.05	$<1^d$	5.0

Table 4.4 continued...

Patient ^a	Age in months		V _β 8/10 ⁴ cells		HIV-1/10 ⁴ cells		% CD14 ⁺ cells ^b
			CD4 ⁺ T	CD14 ⁺	CD4 ⁺ T	CD14 ⁺	
SHFI	588	17.0	10	1	10	<1	4.0
	1279	34.0	ND	ND	1	<1 ^d	8.0
	1539	44.0	ND	ND	1	0.4	6.0
SHJO	530	5.8	40	1	50	<1	7.0
	1162	22.0	ND	ND	1.4	<1 ^c	6.0
NIBA	1518	4.3	ND	ND	20	1	5.0
REGR	1278	1.0	ND	ND	10	<1	3.0
	1443	7.5	6	1	0.2	<1 ^d	12.0

^a designated by initials^b percent of total PBMC according to flow cytometry analysis^c not in the linear range^d less than 1 copy of HIV-1 detected in 2.5 X 10⁴ cells^e less than 1 copy of HIV-1 detected in 5 X 10⁴ cells

ND, not done; NA, not available; bold letters, samples also amplified with M667-AA55 primers

Table 4.5. Detection of $V_{\beta}8$ and HIV-1 sequences in DNA from $CD14^+$ monocytes and $CD4^+$ T lymphocytes of infected children 2-13 years of age

Patient ^a	Age in years	$V_{\beta}8/10^4$ cells		HIV-1/ 10^4 cells		% $CD14^+$ cells ^b
		$CD4^+$ T	$CD14^+$	$CD4^+$ T	$CD14^+$	
MIST ^c 1072	10.7	ND	ND	3	<1	8.0
CHST 1224	7.1	12	<1	1.5	<1^d	8.0
JOST 1438	7.5	ND	ND	0.2	<1 ^d	11.0
NIHI ^c 1103	10.8	ND	ND	$\geq 100^e$	<1	9.0
1551	12.0	ND	ND	0.1	<1 ^f	8.0
JOLE 432	5.1	6	2	1.5	<1	10.0
1254	7.6	ND	ND	5	1	1.9
JASM 1169	7.7	ND	ND	1.0	<1 ^d	14.0
1194	7.8	14	1	3	1	12.0
DABU 1087	4.3	ND	ND	$\geq 250^e$	<1	6.0
TRGR 1117	3.5	ND	ND	$\geq 1000^e$	<1	2.0

^a designated by initials

^b percent of total PBMC according to flow cytometry analysis

^c patients infected by contaminated blood product at birth

^d less than 1 copy of HIV-1 detected in 2.5×10^4 cells

^e not in the linear range

^f less than 1 copy of HIV-1 detected in 5×10^4 cells

ND, not done; bold letters, samples also amplified with M667-AA55 primers

HIV-1 infection of peripheral blood monocytes. Results from analysis of 37 blood samples, from 12 vertically infected children followed longitudinally from birth, and from a sample of an 18 months old child (TEWI), are shown in Table 4.4. Four samples belonged to neonates (<2 months old). In 16 out of 38 cases, patients were asymptomatic (CDC class N-A). Except for TEWI, from whom information was not available, 5 children had virus detected in their PBMC by PCR at the time of birth, suggesting in utero infection. Eleven samples belonged to children who had not been followed from birth (Table 4.5). Other than 2 patients who were infected at birth by contaminated blood product, all others acquired HIV-1 from their infected mothers. There was only 1 sample in this group from an asymptomatic patient (MIST). We also analyzed 17 blood samples from infected adults, transmitting and non-transmitting mothers (Table 4.3). Among the non-transmitters, 5 received zidovudine (ZDV) to prevent vertical transmission of HIV-1, according to the ACTG protocol 076. Maternal samples were used as a positive control of virus detection, and for comparison of viral tropism between the mother-child pair. Three of the 5 mothers presenting clinical evidence of HIV-1 infection, transmitted the virus to their children.

Overall, the extensive analysis performed was insufficient to demonstrate unequivocally the presence of HIV-1 *gag-pol* or *env* sequences in blood monocytes of children (and adults) of any age, or disease stage. Representative data is

shown in figure 4.1. When viral sequences were detected in the monocyte population (21/66 samples), 2-300 times more copies of HIV-1 were detected in the CD4⁺ T lymphocytes selected from the same blood sample (Tables 4.3-4.5). The presence of contaminating T cells, represented by detection of V_β8 products, could account for the detection of HIV-1 in monocytes in 13/21 cases (TEBU, HAST, MEST [31.0 mo], DEWA [11.0 mo], DIKA [9.5 mo], SAFR [8.3, 9.5, and 11.8 mo], JERO [1.0, 5.9, and 8.0 mo], JALA [2.0 mo], and JASM [7.8 yo]). V_β8 amplification was not performed in the remaining 8 cases, due to insufficient DNA availability, however, ≤ 1 copy of HIV-1 was detected per 10⁴ monocytes. However, based on the results of V_β8 analysis, viral amplification in these 8 cases could very likely be due to infection of contaminating CD4⁺ T lymphocytes.

An important factor is the distribution of V_β8 family of TCR in HIV-1 infected individuals. In the normal population, V_β8 rearrangements are detected in 3-26% of peripheral T lymphocytes, with similar occurrence in both CD4⁺ and CD8⁺ T cells (84, 148). It has been reported that HIV-1 infects preferentially CD4⁺ T cells expressing rearranged V_β12 TCR (179). We noticed that V_β8 rearrangements comprised only 0.08-0.8% of the CD4⁺ T cell population in our cohort. Detection of these cells in the monocyte population at a level that could explain the presence of amplified virus, strengthens the

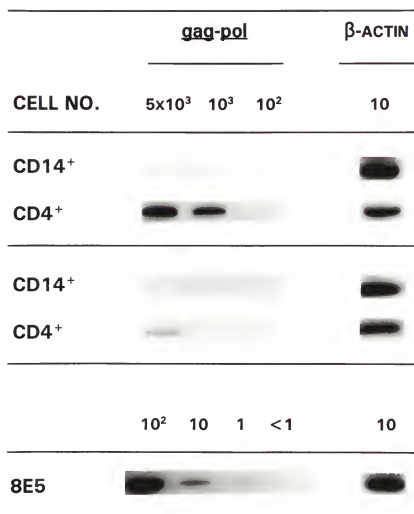


Figure 4.1. HIV-1 DNA in PBMC. Serial dilutions of DNA equivalent to 5×10^3 to 10^2 cells were obtained from CD14⁺ monocytes and CD4⁺ T lymphocytes selected from 2 separate individuals by using immunoaffinity magnetic beads (upper pannel). Serial dilutions of DNA equivalent to 10^2 to <1 cell were obtained from 8E5 T cell line (lower pannel). DNA from each cell type was amplified with *gag-pol* primers. Products were electrophoresed in 1.0% agarose gels and transferred to Nytran membranes. The DNA equivalent of 10 cells from each cell type was also amplified with primers for β -actin. Double-stranded DNA probes to detect envelope and actin sequences were labelled by random priming with α [³²P]dATP, washed, and exposed to X-ray film.

results that CD14⁺ cells do not comprise a major virus reservoir.

If it were true that macrophage-tropic variants initiate HIV-1 infection after vertical transmission (196), we would expect to detect virus in monocytes at least during the acute phase of infection. During acute HIV-1 disease, high levels of virus replication take place and peak levels of viremia are reached during this stage. As an efficient immune response develops, viral burden is downregulated. The acute phase of infection lasts 3-6 months in adult individuals (106). Vertically infected neonates/infants are a good model for acute infection, since a neonate can not be infected for longer than 9 months. Frequently infection occurs later during gestation, close to the time of birth (negative HIV-1 PCR at birth). Four neonates (\leq 2 months of age), 3 of whom were HIV-1 PCR-positive at birth, had samples analyzed. In 2 cases, there was <1 copy of HIV-1 per 10^4 or 5×10^4 monocytes (REGR, JOFO), while in the other 2, 75-100 times more copies of HIV-1 were detected in the CD4⁺ T cells than in the monocytes (JALA, JERO). Furthermore, V₈ sequences were detected in the monocytes at a level that could explain the presence of HIV-1 in the monocyte population. Interestingly, previous experiments performed in our laboratory were able to show HIV-1 in monocytes from 2 neonates, SHFI and SHJO. V₈ sequences were not detected at the level of 10^4 CD14⁺ cells. One copy of HIV-1 per 10 CD4⁺ T cells and $1/10^3$ monocytes were identified

in SHFI's sample; SHJO had equally infected CD4⁺ T cells and monocytes, at 1/10². Unfortunately, there is no DNA available from these patients at that time, and the experiment could not be repeated. When 2 later samples from each patient were analyzed, virus was not detected in 10⁴ to 5 X 10⁴ monocytes; in a sample from SHFI at 44 months of age, there was 0.4 copy of HIV-1 per 10⁴ monocytes. Although V_β8 amplification was not performed in this specific sample, the low level of detection could be attributed to contaminating infected CD4⁺T cells with other TCR rearrangements. The 2 previous results in which virus was detected in monocytes, could represent a brief window of time when high levels of virus replication occurs in these cells, before decreasing abruptly to basically undetectable levels. Otherwise, it is possible that these patients had low levels of rearranged V_β8 TCR at that time, and that the absence of detection of this family in monocytes did not represent a good marker for monocyte purity.

Maybe virus was not detected in monocytes because of a low level of cell associated HIV-1 in these individuals. This could not explain our results because even when as much as 3% of patient CD4⁺ T lymphocytes were infected, monocytes only contained 0.01 to 0.04% of detectable viral sequences, a fact that could be explained by the presence of T cells in this population. Because viral DNA could not be detected in maternal blood samples either, we excluded the possibility that this finding was specific to the pediatric population.

Finally, it is possible that the HIV-1 patients we studied presented severe monocytopenia, which could explain why virus was not detected in their cells. This hypothesis could not be confirmed because HIV-infected children of all ages had normal numbers of monocytes in their peripheral blood, 7.6% (\pm 4.4) in children 0-2 years of age, and 8.4% (\pm 4.2) in children older than 2 years of age.

Strong-stop DNA. Peripheral blood monocytes have few to none detectable HIV-1 *gag-pol* or *env* DNA sequences. We reasoned that if monocytes were susceptible to HIV-1 infection but virus replication only occurred after cells started the differentiation process into macrophages, this could explain why we did not detect HIV-1 DNA in these cells. Previous reports showed partial DNA products of reverse transcriptase in quiescent peripheral blood lymphocytes, whereas full reverse transcription did not occur until mitogenic stimulation (214). It has also been shown that in culture incomplete DNA species are detected in non-proliferating monocytes (171). Using an adaptation of the technique described by Zack (214), 25 to 100 ng DNA of CD14⁺ monocytes and CD4⁺ T lymphocytes from 8 patients were PCR amplified with M667-AA55 primers, directed towards the strong-stop sequence of HIV-1 LTR (Fig. 4.2). The difference in our assay was the use of a radiolabelled oligonucleotide probe instead of radiolabelling the amplification primers.

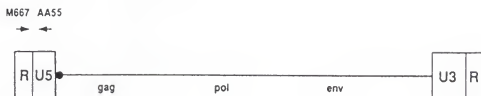


Figure 4.2. Oligonucleotide primers specific for LTR strong-stop DNA. Location and orientation of primers M667 (nucleotides 496-516) and AA55 (nucleotides 612-635) are shown in relation to HIV-1 RNA genome. Open boxes represent the LTRs and the solid circle represents the primer binding site (PBS), located in nucleotides 637-651. tRNA primer binds to PBS to start minus-strand polymerization to the end of the R region. R-U5 DNA is made, which will hybridize to the R region of the 3' RNA LTR. The R-U5 RNA is degraded by reverse transcriptase, which has RNase H activity, during the reverse transcription process (Modified from Ref. 214).

Seven of the 8 patients from whom samples were amplified with these primers are shown in Tables 4.3-4.5. The eighth patient was a 3 month-old infant from whom only strong-stop DNA amplification was performed, therefore not included in the tables. Among the 4 children <2 years of age, 2 demonstrated no signs of clinical disease (REGR and CAHA). There were 2 older children, both with symptomatic disease (class B-C), and 2 mothers, 1 transmitter and 1 non-transmitting. Although virus sequences were always detectable in the CD4⁺ T lymphocytes, monocytes from these individuals did not harbor partial reverse transcripts.

Therefore, the conclusion from these series of experiments is that peripheral blood monocytes do not serve as a major reservoir of HIV-1 infection.

HIV-1 cultures and genotyping. Preliminary data from our laboratory show that macrophage-tropic variants are detectable in PBMC samples from patients of our cohort. Briefly, the assay consists of cocultivating patient's PBMC with uninfected PBMC, and the virus stock produced is used to infect primary monocyte-derived-macrophages (MDM). Macrophage-tropic virus was detected in a sample from a transmitting mother (TEBU). Interestingly, her child (SAFR) did not present viruses with such characteristics. The same was true for 2 other children, CHST and MEST. More samples need to be analyzed for further conclusions.

Genotyping, to determine if viral sequences with characteristics of macrophage-tropism are found in the blood of our patients, is currently being done in our laboratory.

These experiments will be important to elucidate why viral DNA is not detected in our cohort at significant levels. Either these patients do not have macrophage-tropic viruses or blood monocytes are truly not infected, which we believe to be the most likely explanation.

Infection of CD4⁺ T lymphocytes in longitudinal and cross-sectional analysis of HIV-1 infected children. Since blood monocytes in the population of children we studied in most cases harbored no detectable HIV-1, we analyzed the pattern of infection in the CD4⁺ T lymphocytes from these individuals. A total of 121 blood samples were studied, 92 of which belonged to 13 vertically infected children who were followed longitudinally from birth (Table 4.1). There were 20 blood samples from 3 children >2 years of age, who were also followed longitudinally, and 9 samples from children 1-13 years of age, which were analyzed cross-sectionally (Tables 4.1 and 4.2). After immunomagnetic selection of CD4⁺ T lymphocytes from total PBMC, DNA from these cells was PCR amplified with *gag-pol* and *env* primers. Cell-associated viral load was calculated by comparison of results to an 8E5 DNA standard curve. In approximately 50% of the cases, DNA from total PBMC was amplified instead and the number of infected

CD4⁺ T cells was calculated according to flow cytometry results of T cell subset analysis.

The 13 children who were followed longitudinally were divided into 3 groups for purpose of analysis, slow progressors, rapid progressors, and indetermined (Table 4.1). Criteria used for classification of patients included result of PCR at the time of birth, clinical and immunological status of the child, and timing when peak levels of cell-associated virus were reached. Groups clustered according to progression of disease were further divided into age groups 0-6 months, 7-12 months, 13-24 months, and 25-72 months, for statistical analysis. Comparison was performed among children within the same age range, with either slow or rapid development of disease. One time point samples were obtained from patients TEWI and MEBE (Table 4.1), not included in the statistical analysis. Representative data is shown in figures 4.3A (slow progressors) and 4.3B (rapid progressors).

The 5 slow progressors (Table 4.6) were HIV-1 PCR negative at birth and asymptomatic (CDC class N-A) until 24 months of age. Peak levels of cell-associated virus were reached around 12 months of age (12.7 ± 2.5). Among the 6 rapid progressors (Table 4.7), 5 had a positive PCR at birth and by 6 months of age, only 1 patient was still asymptomatic, without signs of immunosuppression (SHJO). Excluding the patient from whom we did not have an earlier sample (SAFR), peak levels of cell-associated virus in this group were

A

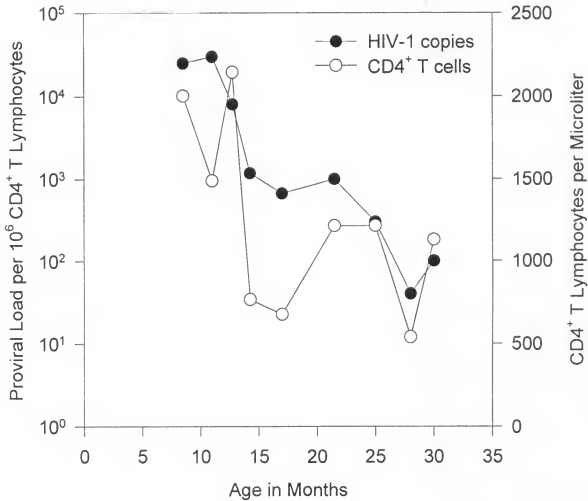


Figure 4.3. HIV-1 proviral copy number and CD4⁺ T cell count in vertically infected children. Longitudinal analysis performed in a slow progressor (A) and in a rapid progressor (B).

Figure 4.3 continued...

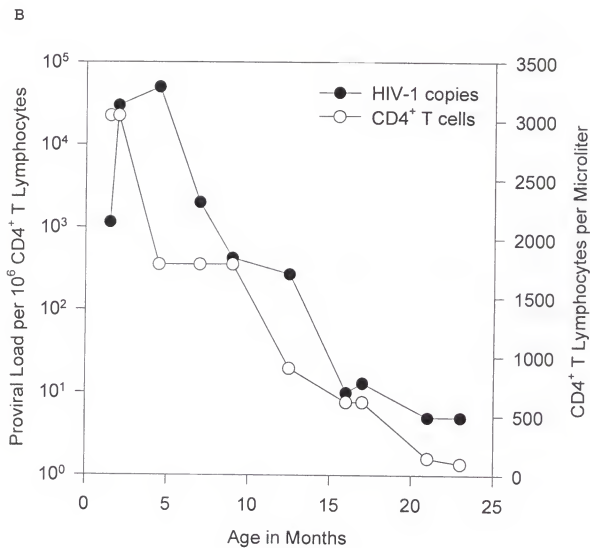


Table 4.6. Characteristics of HIV-1 vertically infected children followed longitudinally from birth, classified as slow progressors^a

Age in months	N ^b	CD4 ⁺ T cells/ μ l	CD8 ⁺ T cells/ μ l	CD4:CD8 ratio	% HLA-DR ⁺ T cells	CDC stage ^c	HIV-1 copies/ 10 ⁶ CD4 ⁺ T cells
0-6	2	3141 (\pm 1172)	1520 (\pm 1059)	2.4 (\pm 0.8)	55	100%	98 (\pm 98)
7-12	9	2692 (\pm 1237)	1995 (\pm 750)	1.5 (\pm 0.5)	16 (\pm 9)	100%	8161 (\pm 11670)
13-24	9	1512 (\pm 654)	2071 (\pm 902)	0.8 (\pm 0.5)	32 (\pm 16)	100%	740 (\pm 542)
25-72	14	887 (\pm 411)	1530 (\pm 998)	0.9 (\pm 0.9)	15 (\pm 6)	21%	136 (\pm 360)

^a results are expressed as mean (\pm standard deviation)

^b number of samples analyzed

^c expressed as percent of asymptomatic individuals (CDC class N-A)

Table 4.7. Characteristics of HIV-1 vertically infected children followed longitudinally from birth, classified as rapid progressors^a

Age in months	N ^b	CD4 ⁺ T cells/ μ l	CD8 ⁺ T cells/ μ l	CD4:CD8 ratio	% HLA-DR ⁺ T cells	CDC stage ^c	HIV-1 copies/ 10 ⁶ CD4 ⁺ T cells
0-6	13	1822 (\pm 748)	1437 (\pm 524)	1.4 (\pm 0.6)	10 (\pm 5)	77%	12636 (\pm 15049)
7-12	7	974 (\pm 580)	1327 (\pm 376)	0.9 (\pm 0.7)	10 (\pm 4)	0%	7670 (\pm 8616)
13-24	12	388 (\pm 317)	707 (\pm 390)	0.5 (\pm 0.4)	19 (\pm 11)	8%	876 (\pm 1065)
25-72	15	515 (\pm 297)	1867 (\pm 2581)	0.5 (\pm 0.3)	19 (\pm 10)	20%	488 (\pm 720)

^a results are expressed as mean (\pm standard deviation)

^b number of samples analyzed

^c expressed as percent of samples from asymptomatic individuals (CDC class N-A)

reached at 3 (± 1.8) months. Rapid progressors were initiated in antiretroviral therapy at an earlier age than slow progressors, 5.6 (± 3.3) months of age versus 15.4 (± 2.6) months of age ($p=0.003$). At this time, 2 patients are still indetermined in respect to disease progression (Table 4.4). Both indetermined children had a negative PCR at the time of birth. For purposes of statistical analysis, these children were not included.

Results of statistical analysis comparing slow versus rapid progressors are shown in Table 4.8. Rapid progressors always had lower CD4 counts than slow progressors, suggesting that these children showed signs of immunosuppression early in life, at or shortly after birth, which persisted until 6 years of age, when analysis was interrupted. Children who were infected close to the time of birth (PCR negative at birth) seemed to have a greater potential to combat the virus, leading to slower development of disease. Likely, these patients had a more developed immune system by the time of the first encounter with the virus. Similar levels of cytotoxic T lymphocytes were detected in both groups, independent of the degree of immunosuppression, except for children 13-24 months of age, when rapid progressors had significantly lower CD8 counts ($p=0.002$). Also at this age, decrease in CD4 counts was most pronounced in rapid progressors, when compared to slow progressors. CD8 decline probably followed as a consequence of the marked reduction in CD4 counts. Inversion of CD4 to CD8

Table 4.8. p Values based on statistical analysis of data in tables 4.6 and 4.7 (slow versus rapid progressors)

Age in months	CD4 ⁺ T cells/ μ l	CD8 ⁺ T cells/ μ l	CD4:CD8 ratio	% HLA-DR ⁺ T cells	CDC stage	HIV-1 copies/ 10 ⁵ CD4 ⁺ T cells
0-6	0.046	0.85	0.038	0.29	1	0.05
7-12	0.005	0.05	0.082	0.18	<0.0001	0.40
13-24	< 0.0001	0.002	0.11	0.12	< 0.0001	0.67
25-72	0.027	0.37	0.064	0.50	1	0.31

bold, significant values

ratio usually occurred between 13 and 24 months of age in both groups.

Signs and symptoms of HIV disease did not show in either group until patients were 6 months old. Between 7 and 24 months of age, however, most rapid progressors presented symptomatic HIV infection while all slow progressors were still asymptomatic ($p < 0.0001$). It was not until 2 years of age when slow progressors started showing symptoms.

Curiously, there was no difference in the level of infected CD4⁺ T lymphocytes in children 0-6 years of age. There was a striking difference in viral load between the groups of slow and rapid progressors at 0-6 months of age (98 [\pm 98] versus 12636 [\pm 15049]). However, the difference was not statistically significant by Mann-Whitney rank sum test ($p = 0.0507$). The small number of slow progressors (2 versus 13) could in part explain these results. Although peak levels of cell-associated virus were detected earlier in rapid progressors than in slow progressors (3 and 12 months, respectively), levels of viremia were still similar between the 2 groups. At 1 year of age, viral load was still relatively high in rapid progressors, in the process of declining, before entering a stable, low level phase of viremia.

There was also no difference in the level of activation of total T lymphocytes among patients from both groups, measured through detection of HLA-DR marker by flow cytometry

analysis. The similar results detected in both slow and rapid progressors suggest that lymphocyte activation of either CD4⁺ or CD8⁺ T cells is not a factor in development of disease.

We then analyzed patients >2 years of age, who had not been followed from birth (Table 4.2). This analysis included 3 patients who were followed longitudinally, and 5 patients from whom only 1 or 2 blood samples were examined. Because in most cases PCR result at birth, timing of peak viremia and disease stage throughout the first year of life were unknown, these children were classified into asymptomatic (CDC class A) or symptomatic (CDC class B-C) categories, according to the time when first sample was obtained. Children 2-6 years of age, A or B-C, did not differ in respect to CD4 or CD8 counts, or in the level of infected CD4⁺ T cells (Table 4.9). This result is different from what we found in slow and rapid progressors 2-6 years of age, CD4 counts being significantly lower in rapid progressors. We expected asymptomatic patients to be similar to slow progressors in most aspects. The small sample number in the asymptomatic group 2-6 years of age could explain the difference.

Children 6-13 years of age were similar in respect to the number of activated (HLA-DR⁺) T cells and there was no difference in the level of infected CD4⁺ T lymphocytes. However, CD4⁺ and CD8⁺ T cell counts continued to be higher in asymptomatic patients, even at this older age (Table 4.9).

Table 4.9. Characteristics of HIV-1 infected children 2-13 years of age, classified according to the disease stage^a

CDC stage	N ^b	CD4 ⁺ T cells/ μ l	CD8 ⁺ T cells/ μ l	CD4:CD8 ratio	% HLA-DR ⁺ T cells	HIV-1 copies/ 10 ⁶ CD4 ⁺ T cells
2-6 years of age						
A	1	1059	2744	0.4	NA	182
B-C	9	852 (\pm 301)	1589 (\pm 671)	0.6 (\pm 0.3)	24 (\pm 11)	728 (\pm 652)
p ^c		0.49	0.3	0.49	NA	0.6
6-13 years of age						
A	6	813 (\pm 138)	2579 (\pm 1000)	0.4 (\pm 0.4)	25 (\pm 6)	282 (\pm 178)
B-C	11	462 (\pm 301)	891 (\pm 402)	0.5 (\pm 0.3)	28 (\pm 11)	445 (\pm 623)
p ^c		0.039	0.0002	0.54	0.74	0.65

^a results are expressed as mean (\pm standard deviation), except for 2-6 years of age, A, where results from 1 sample only are represented

^b number of samples analyzed

^c p value

NA, not available

HIV-1 infection of CD45RA and CD45RO subpopulations of CD4⁺ T lymphocytes. Previous reports showed that in adults the memory CD45RO subtype of CD4⁺ T lymphocytes is preferentially infected over the naive CD45RA cells (168). Functional abnormalities seen in infected individuals, as for example a defect in the ability to respond to soluble antigens, could be a direct consequence of the preferential infection of memory T cells. To investigate infection of CD4⁺ T cell subtypes in the pediatric population, 11 blood samples from 10 children were analyzed (Table 4.10). Using immunomagnetic beads, CD14⁺ monocytes were selected initially from total PBMC, because besides coexpressing the CD4 surface antigen, a subpopulation of monocytes also expresses the CD45RO ligand (1). CD4⁺ T lymphocytes were selected with detachable immunoaffinity beads from the CD14-depleted fraction. Cells of the memory (CD45RO) and naive (CD45RA) phenotypes 95% enriched according to flow cytometry analysis, were obtained by immunomagnetic selection from CD4⁺ T lymphocytes. PCR amplification with *env* primers was performed in equal concentrations of DNA from both CD45RA and CD45RO cells, ranging from 100 to 1,000 ng within the same blood sample.

Quantitation of HIV DNA in T cell subsets from 4 children 2 to 9 years old, revealed 4- to 10-fold more HIV-1 DNA within the CD4⁺ memory T cells than in the naive lymphocytes. Only the oldest patient in this group (MIST), who was infected at birth by contaminated blood product, was still asymptomatic.

Table 4.10. HIV-1 infected CD45RA and CD45RO CD4⁺ T cells

Patient	Age in months	Infected cells/10 ⁵	
		CD45RA	CD45RO
0-2 years of age			
REGR 1278	1.0	<1 ^a	100
SHJO 494	1.3	<1	1
JOFO 1516	1.8	80	40
NIBA 1518	4.3	30	160
JOSH 1414	15.5	30	<1 ^a
	1536 20.0	70	70
TEWI 1547	18.0	10	<1 ^b
2-6 years of age			
JERO 1537	29.0	20	80
MEST 1555	72.0	<1 ^b	10
6-13 years of age			
ALGA 504	93.0	<1 ^a	20
MIST 348	104.0	1	10

^a Less than 1 copy of HIV-1 detected in 10⁴ cells^b Less than 1 copy of HIV-1 detected in 5 X 10⁴ cells

These results are in agreement with the published data in infected adult individuals (168).

Children younger than 2 years of age can have up to 80% of the CD4⁺ T cells comprised of the naive phenotype (94). The 6 patients analyzed in this group acquired HIV-1 from their infected mothers and presented no symptoms (CDC class N-A) at the time. Among the 3 neonates (<2 months of age) studied, 2 had >10-fold more virus detected in the CD45RO population. The third child (JOFO) had 2-fold more HIV-1 DNA present in the CD45RA naive T cells than in the CD45RO lymphocytes. This patient was infected in utero, according to a positive HIV-1 PCR result at the time of birth. However, timing of maternal transmission did not seem to associate with preferential infection of naive T cells, since this phenomenon was not true for the patient SHJO who also had a positive PCR at birth.

The other 3 infants were infected perinatally and did not differ from each other in respect to their clinical characteristics. Only 1 patient (NIBA) had greater viral load in the memory T cells; in the other 2 children, equal amounts to >10-fold more virus were present in the naive cells.

From this section of the study we concluded that in children >2 years of age, CD4⁺ memory T cells are the main subpopulation infected by HIV-1, as seen in adults. However, in younger children either lymphocyte subtype can harbor the virus. This finding does not seem to correlate to timing of infection or clinical symptomatology. It will be interesting

to determine if viruses that replicate during acute and initial stage of chronic infection are genotypically different from viruses present during the established chronic phase of HIV-disease. There could be a correlation between preferential CD45RA or CD45RO infection, and progression of disease.

Discussion

HIV-1 DNA in blood monocytes. It is well established that HIV-1 can replicate in tissue macrophages, and that the virus strain most commonly found in these long-lived cells is not cytopathic, therefore, macrophages can serve the function of HIV-1 reservoir. Infection of macrophages has also been held responsible for CNS pathogenesis. A topic less well agreed upon concerns the presence of HIV-1 in the macrophage precursor cells, namely, blood monocytes. Some authors have reported undetectable to low level infection of these cells in adults (116, 124), although others claim that virus is present in approximately 75% of cells (9). In many instances, it is unclear how pure was the population of monocytes assayed; residual infected CD4⁺ T lymphocytes could account for detection of HIV-1 in selected monocytes (11). Furthermore, although several researchers approached infection of monocytes in adult individuals with a variety of symptoms and under different therapeutic regimens, the field is still open in respect to monocyte infection in the pediatric population. Most studies involving children assayed susceptibility of cord

blood or neonatal monocytes to HIV-1 infection in culture (98, 185). In view of the contradictory reports regarding monocyte infection and the unexplored issue about the presence of HIV-1 in pediatric monocytes, we performed an extensive analysis to elucidate this subject.

For most instances, significant levels of HIV-1 DNA could not be detected in monocytes from our cohort. These results are in agreement with Massari et al (116), who detected HIV sequences by PCR only in 2 out of 53 adult blood samples analyzed, in contrast to 53/53 PCR-positive T lymphocytes. The isolation technique used and most important, the ability to check for T cell contamination in the monocyte fraction, provide strength to our data.

The technique we used to separate monocytes was based on the expression of CD14 surface antigen by these cells. If this molecule is downregulated by HIV-1 infection, a possible explanation for our results would be a biased selection of uninfected cells. This does not seem to be the case. Previous work showed that in the contrary, expression of membrane-bound CD14 on peripheral blood monocytes was significantly increased during the course of HIV infection, and in all stages of disease (137). Data from our laboratory showed no difference of CD14 expression in monocytes from infected versus uninfected individuals, by flow cytometry analysis.

Some studies have reported a variation in the detection of HIV-1 by *gag* and *env* primers, and inefficiency to amplify

LTR sequences in monocytes (96, 124). Our PCR assay is sensitive to the level of detection of 1-5 copies of HIV-1 with both *env* and *gag-pol* primers. Two sets of primers were employed for amplification in 12/66 samples (18.2%), and either *gag-pol* (20/66, 30.3%) or *env* (34/66, 51.5%) primers were used randomly in the remaining cases. In addition, in 8/66 (12%) samples a third set of primers was used which would detect strong-stop DNA, in case of latent infection of these cells.

An important issue to deal with is the fact that in 21/66 samples analyzed, <1-4 copies of HIV-1 could be detected per 10^4 monocytes. The presence of infected $CD4^+$ T lymphocytes mixed in this population most likely accounts for the majority of these results, based on TCR detection in the samples. However, there were some ambiguous results. Previous experiments done in our laboratory showed significant levels of infection of monocytes in blood samples from 2 neonates. Several possibilities exist. First, it is possible that for a short period of time viral replication occurs in these cells at high levels, and amplification was done at the correct time to detect such event. Otherwise, if the $V_{\beta}8$ family of TCR represented insignificant percentage of all TCR families at that time, the absence of detection of $V_{\beta}8$ sequences in their monocytes could not be a true reflection of monocyte purity. Finally, we have altered our procedure for sample manipulation. Although we now process blood samples

immediately after collection, and rarely within a maximum of 24 hours, previously the samples were not processed within this time frame. Sometimes, 2-3 days would elapse before sample processing started. One could argue that the differentiation process could have initiated in these monocytes as blood waited to be separated, therefore causing these cells to become susceptible and eventually infected by HIV-1.

Inconclusive results were attributable to other samples, when viral load in monocytes was only 2-10 fold lower than in CD4⁺ T lymphocytes. Samples from MEST at 31 months of age and HAST (mother) were processed during the same time as the samples from 2 neonates who had detectable virus in monocytes. Although contamination of monocytes by CD4⁺ T lymphocytes could account for viral amplification, same reasoning as for the 2 neonates would apply. V₈ analysis could not be performed in the samples from DEWA at 21.5 months of age, and JOLE 7.6 years old. Therefore, the possibility of T cell contamination could not be ruled out. Samples from the same patients analyzed before and after this date indicated that their monocytes do not harbor HIV. Blood sample from SHFI at 44 months of age was not analyzed for TCR sequences. Two previous samples from the same patient had undetectable HIV-1 DNA in monocytes, which would suggest the likeliness of uninfected cells. However, this is one of the two patients who previously had virus detected in monocytes as a neonate. A

definite conclusion, therefore, can not be made at this time about infection of this patients' monocytes. In the case of JASM at 7.8 years of age and TEBU (mother), detection of $V_{\beta 8}$ sequences in their monocytes and inability to amplify strong-stop DNA in the same samples, suggest that these cells are not infected.

It is relevant to mention that preliminary studies in our laboratory were able to detect macrophage-tropic viruses in a fraction of patients from this cohort. Although still premature to make final conclusions, it appears that virus with this phenotypic characteristic is present in $CD4^+$ T lymphocytes, and can exist in the absence of peripheral blood monocyte infection.

In conclusion, based on the strong evidence from this study, we conclude that peripheral blood monocytes are not a main reservoir of HIV-1 in the pediatric population and their mothers. These results are important for therapeutic and vaccine development, in the respect that $CD4^+$ T lymphocytes are the major infected cell type and virus reservoir in PBMC, and should be the main target in the combat of HIV-1.

Infection of $CD4^+$ T lymphocytes. We performed detailed studies in HIV-1 infected children prospectively followed from birth or analyzed cross-sectionally. We noticed two patterns of disease development in vertically infected children followed from birth. In one group peak levels of viremia were reached in the first few months of life (around 3 months of

age), while the other infants did not reach peak viremia until close to 1 year of age. Peak levels of viremia could be as high in the slow as in the rapid progressors. Peak viremia was reached abruptly, was of short duration, and also dropped abruptly in rapid progressors. The same fast decline was observed in slow progressors, however, we can not comment on the pattern of the slope before reaching the peak, since these children many times returned to our clinic only around 6-8 months of age. Passed the acute phase of infection, levels of cell-associated virus were downmodulated to similar extent in both groups. Interestingly, comparison of viral burden in CD4⁺ T lymphocytes between patients of the same age, did not differ among the groups, whether slow or rapid progressors, or symptomatic or non-symptomatic (children who were evaluated cross-sectionally). These results differ from a previous study, where symptomatic children had an average 20-fold higher frequency of infected CD4⁺ T cells (48). It is possible that the new therapeutic approaches, including the use of protease inhibitors, combination antiviral drugs, and early start of treatment, could account for the low level of intracellular viral load in sicker children. We also did not see a different pattern of detection of free virus in the plasma of these patients, according to p24 antigen measurement.

Different than viral burden, children with rapid development of disease always had significantly lower CD4 counts, from birth to 6 years of age. Although signs of

immunosuppression were detected soon after birth in these patients, they were asymptomatic during the first 6 months of life, like the slow progressors. Symptoms usually developed before 1 year of age in the rapid progressors, while children in the other group were asymptomatic until they turned 2 years old. From 2-6 years of age, the 2 groups were similar in this respect.

The 5 slow progressors were PCR negative at the time of birth, while 5/6 rapid progressors were infected in utero. Timing of infection, therefore, correlated to the pattern of disease progression. A likely explanation is that children infected perinatally have a more mature and competent immune system, resulting in better control of infection. CD4 counts were also lower in symptomatic children between 6 and 13 years of age; no difference was detected between children 2 to 6 years of age, who were analyzed cross-sectionally. We expected to find similar results in this group to the observed in patients followed longitudinally from birth, of the same age. Because there was only 1 sample from asymptomatic children in the cross-sectional analysis, this result may not reflect a good representation of the group. Although we can not draw any conclusion about the degree of CTL (cytotoxic T lymphocyte) response in these patients, CD8 counts were similar in both slow and rapid progressors throughout this study, except for the period of 13-24 months, when CD8s were lower in rapid progressors. When symptomatic and non-symptomatic children 6-

13 years of age were compared, a significant difference was detected in the levels of CD8 cells. These results are similar to what is observed in infected adults in respect to disease development, where a decrease in CD8 cells is only apparent during the final stages of disease.

We extended our analysis into determining which subpopulation of CD4⁺ T lymphocytes is preferentially infected in pediatric patients. As seen in adult individuals (168), the CD45RO memory T cells consist the main virus reservoir in children older than 2 years of age. However, CD45RA and CD45RO may be equally infected in infants. At this age, around 80% of the lymphocytes belong to the naive subtype. Furthermore, high levels of viral replication are taking place in these children, before they enter the chronic stage of disease. An association of these factors could explain why naive cells, even if less susceptible to HIV-1, may still be infected to the same extent as the CD45RO lymphocytes.

CHAPTER 5 CONCLUSION

The AIDS epidemic represents one of the greatest medical challenges in human history and still, education is the only means to effectively control the disease. Although the threat that HIV imposes to women's health is high, the impact of HIV infection on the female population is perhaps the most inadequately studied aspect of the epidemic. In sub-Saharan Africa, women comprise 50% of the AIDS cases, and in the United States, infection among women shows the greatest increase in reported cases of AIDS (60). Relatively little has been published about the medical, psychological, and social consequences of HIV disease in women. Small numbers of HIV-infected women have been involved in clinical trials. The risks and benefits of therapeutic interventions are mainly concluded from studies in the male population, and may not be applicable to women. Most HIV-infected women are of childbearing age, and the number of children affected by this disease continues to grow, to an estimated number of 10 million cases worldwide by the year 2000.

The aims of my study were to analyze with precision specific aspects of mother and child HIV-1 disease, including the role of blood monocytes in maternal-fetal HIV-1 infection,

and effect of cell-associated viral load in transmission and development of pediatric disease.

Maternal transmission of HIV-1

Approximately 85% of women with AIDS are at childbearing age (28), therefore, as the number of HIV infection in women rises, so does the number of infected infants. Multiple factors are associated with vertical transmission of the virus, of both maternal and fetal origin. A clear relationship exists between transmission and immunological state of the mother, biological characteristics of the virus, and obstetrical factors. Immunological status is altered by pregnancy, reflected by a change in lymphocyte response to antigens and in a decrease in both relative and absolute CD4⁺ T cell counts. It is not entirely clear, however, in what manner T cell function is compromised in pregnancy. Factors that may contribute to the immunosuppression of pregnancy are increased levels of total steroids and other pregnancy specific hormones, like human chorionic gonadotrophin. Although it has not been demonstrated, these effects could potentially lead to a faster progression of disease in the pregnant woman. However, nowadays most infected pregnant women are asymptomatic and they learn about their HIV status as part of routine pregnancy screening tests. Besides, the majority of infected mothers receive treatment to prevent transmission. These factors could explain why exacerbation of disease during

pregnancy is not noticeable, when compared to non-pregnant infected women.

This study addressed several aspects of maternal and fetal clinical and virological status, and their association with vertical transmission of HIV-1. Influence of maternal age, race, mode of delivery, levels of CD4⁺T lymphocytes and CD4 to CD8 ratios in transmission were analyzed. Study of these characteristics in the absence of antiviral therapy revealed results concordant to previously published data, where lower CD4 counts were associated with increased risk of transmission. We further extended our analysis into examining the effect of viral load in transmission. Few studies have addressed this issue. Maternal plasma RNA levels, measured by quantitative competitive PCR, are significantly related to the chances of an infected woman transmit HIV-1 to her child, according to one group's report (64). Another group, using the same assay, reported no effect of high viral load in transmission (95). Studies on the influence of plasma viremia according to levels of maternal p24 antigen, have shown contradictory results (95, 119, 163). In our analysis, there was a significant positive relationship between transmission and detectable levels of antigenemia. Our analysis revealed a direct association between cell-associated viral load and transmission of HIV-1 to the child. Similar results were also obtained in studies performed by another group (155). These results are important because it has not yet been established

how maternal transmission to the child takes place, if through cell-free or cell-associated virus, or by both means. Our study establishes that the major factors in vertical transmission of HIV-1 are, without doubt, maternal viral load and CD4 counts.

As reported by the ACTG protocol 076 study group (36), in our cohort there was a similar reduction in maternal-infant transmission of HIV-1 with the use of ZDV. Because it is unclear how ZDV causes such an impact in maternal transmission of HIV-1, we evaluated the effect of antiretroviral therapy in maternal proviral load, and its correlation with vertical transmission. We expected to find that ZDV associated decrease in transmission was a consequence of the reduction in viral load. Surprisingly, this was not the case. In treated mothers, transmission occurred independent of the level of maternal virus.

This study defines several aspects of the pathogenesis of mother-to-child transmission of HIV-1, and it can have a major impact in prevention of transmission. Maternal proviral load should be used as a marker of risk of transmission in the absence of therapy. This is very important in third world countries, where ZDV therapy is not readily available to the general population. Pregnant women with high proviral load should preferentially receive treatment, since the risk of transmission in this group (80%) is significantly higher than

in women with less than 100 proviral copies per million CD4⁺ T cells (23%).

It was also clear from this work that ZDV does not affect the level of viremia in infected pregnant women. It is a crucial issue to determine how the drug acts so that more efficient therapy regimens can be available.

Many issues are still unresolved in maternal-fetal transmission of HIV-1. So much is known about HIV but still the most important points, which include prevention of transmission and cure of infection, stay a puzzle.

Future directions. This work does not stop here. At the completion of my PhD I will return to Brazil and continue research in this field. Among the "unsolved mysteries" of vertical transmission of HIV-1, there is a question concerning transmission in poor countries, where factors like protein deficiency, parasitic infections and tropical diseases, could play a role in the risk of a mother transmitting infection to her child. Although some studies have been conducted in African countries, very little is known of this subject in South America, specifically in Brazil.

Importance of studying the population of infected women in Brazil is as follows: (1) Brazil is a large country, about the size of the United States, and it ranks second in the total number of AIDS cases in the world. (2) The principal viral subtype detected in Brazil, clade B, although the same as the primary American subtype, is different in amino acid

sequences of the V3 loop, the major neutralizing domain. Another subtype is also detected in Brazil, clade F, which is also present only in Romania. How the changes in the amino acid sequence of subtype B, and subtype F affect the risk of vertical transmission of HIV-1 has not been studied. (3) Because in the United States and Europe most infected pregnant women take ZDV according to the ACTG 076 protocol to prevent transmission to the child, trials can not be conducted to determine which one of the 3 arms of the therapy affects maternal transmission. In countries like Brazil, the high price of intravenous ZDV makes the drug inaccessible to the general population, and variations of ZDV therapy are beginning to be put in place. Results of these trials will be important for identifying the mechanism of action of ZDV in the reduction of vertical transmission. (4) Infection of the child can occur through breast-feeding. Although it is recommended that HIV-infected mothers do not breast-feed their infants in the United States and Europe, in third world countries the issue is not so easy to deal with. Mortality for non-breast-fed infants is 2 to 5 times higher than for breast-fed infants in developing countries. Therefore, suppressing breast-feeding for all infants born to HIV-1-seropositive mothers could cause a marked increase in overall infant mortality. Undoubtedly, this additional factor in vertical transmission of HIV-1 in Brazil will be important to be investigated.

HIV-1 Infection of Blood CD14⁺ Monocytes and CD4⁺ T Lymphocytes

The second part of this study focussed on clinical and virological markers of progression of pediatric HIV-1 infection. Early recognition and treatment of pediatric HIV infection has affected its clinical course. Although the onset of clinical manifestations of HIV in vertically infected children varies, it is estimated that in over 80% of cases these children will have signs of disease before 36 months of age (61, 141). Children who acquire the infection from their HIV-positive mothers seem to fall into two distinct categories, according to progression of disease. One group will have early presentation of HIV disease, quick progress, and opportunistic infections are the major cause of death in this population. An earlier study (1989) reported that the median survival time for children in whom symptoms of clinical disease appeared before 1 year of age was 24.8 months, while in children who were asymptomatic, the median survival was 6 years (176). A more recent study detected a median survival of 8 years in asymptomatic children (193).

We followed longitudinally from birth children who were vertically infected with HIV-1, to evaluate factors that affected development of disease in view of the more aggressive therapeutic intervention that is being used nowadays. Our first goal was to determine with certainty the role of blood monocytes in acute infection and in disease outcome. We

developed a highly sensitive method to determine the level of purity of selected monocytes. This step was crucial in our investigation because of the highly controversial issue of monocyte susceptibility to HIV-1 infection. After extensive and detailed analysis we determined that monocytes do not comprise a major virus reservoir at any stage of disease in vertically infected children or in their mothers.

These results have important implications in the immunopathogenesis of HIV transmission and disease evolution. Until now, monocytes were believed to be the primary cells involved in maternal-fetal transmission of HIV-1, based on *in vitro* experiments which showed cord blood and neonatal monocytes as more susceptible to infection than both adult cells and the child's T cells (98, 185). This finding helps to clarify the issue of monocyte infection and establishes that CD4⁺ T lymphocyte should be the main target in the combat of HIV-1 transmission, since the role of blood monocytes, if any, is not significant.

We also studied the pattern of disease development in these children. Most studies detected a direct correlation between the severity of disease presentation and the status of the child at birth, determined by HIV-1 culture or PCR. We classified our population primarily based on progression of disease, because sensitivity of PCR can vary in different laboratories. For example, we reclassified 2 children as PCR

positive at birth, different from the clinical laboratory where no virus was detected in their blood at the same date.

Five of the 6 rapid progressors had a positive PCR at birth, and all 5 slow progressors were negative by PCR when they were born. Rapid progressors showed signs of immunosuppression and disease symptoms early on, usually before the first year of life, in contrast to the group which was asymptomatic until 2 years of age. From 2 to 6 years of age, there was no difference in the stage of disease between the rapid and slow progressor groups. These results are extremely important because they demonstrate that although rapid progressors are more prone to acquire infections and develop AIDS faster, the improved therapeutic regimens are expanding the life span of these children significantly, to the extent that when they reach 2 years of age the outcome is equal in both groups. Therapy is very effective in sicker patients. However, it can not be concluded from this study that if treatment were to be started early in the slow progressors, even in the absence of symptoms, disease development and expectancy of life would improve in these children. In our study population, only one patient, a rapid progressor, deceased while being followed.

The study in older children comprised of cross-sectional and longitudinal observations in patients not followed from birth. In the group of children 6-13 years of age, there were only 2 asymptomatic children. One of these children was

infected with contaminated blood product at birth while the other one, who was vertically infected, was a long-term survivor whose mother was also classified this way. These results suggest that the majority of children ≥ 6 years of age present symptoms, independent of disease progression during the first 2 years of life. Since survival in infected children has improved dramatically during infancy, our goal should be to expand the life span of children who are entering adolescence. Perhaps with early therapeutic intervention, not only for symptomatic children but also in pediatric patients still free of signs of disease, the goal of providing both groups with longer and better life could be reached.

Future directions. We have shown that blood monocytes are not a main reservoir of HIV-1. However, to exclude the possibility that our population was different from patients enrolled in other studies, where researchers detected significant levels of virus in monocytes, additional analysis is being performed in our laboratory. Viruses from several individuals of different age and disease stage are being cultured and phenotyped. Preliminary results suggest that macrophage-tropism is a characteristic present in virus from some of our patients. The number of individuals analyzed so far is small for a definitive conclusion. However, we can conclude that macrophage-tropism is a characteristic which can be observed even in the absence of monocyte infection *in vivo*.

Cell-tropism has been linked to the envelope region of HIV-1. To further analyze this aspect, amplification products of the *env* gene will be cloned and sequenced. Because we detected viruses with the macrophage-tropic phenotype in our population, we also expect to identify clones which genotypically express these traits.

Although we did not exam tissue samples from these patients, it is well established that macrophages are infectable. Chronically infected macrophages release only low levels of mature virus progeny *in vitro*, and these cells are relatively resistant to HIV-1-induced cytopathic effects. The ability to sustain low-level persistent infection while harboring large numbers of virus particles has led many researchers to suggest that macrophages serve as virus reservoirs for the persistence and dissemination of HIV *in vivo*. Development of antiviral therapy formulated to target cells of the macrophage lineage should be a primary goal of drug industries. The impact on life span of HIV-1 infected individuals should be immense.

Follow up of children longitudinally characterized in this study will continue, to evaluate with more precision disease outcome later in childhood. Life expectancy in view of improved current therapy will be evaluated.

Another aspect that should be investigated in infected children involves the study of V_{β} families of T cell receptors (TCR). We used the $V_{\beta}8$ family as a way to identify T cell

contamination within selected monocytes, because in the normal population this family is highly represented in peripheral blood (53, 194). Unexpectedly, $V_{\beta}8$ frequently corresponded to <1% of the total $CD4^+$ T cells of HIV-1 infected children.

Recently it was reported that HIV-1 DNA is found specifically concentrated in a small subset (1%-2%) of $CD4$ T cells in many infected patients (50). This subset expressed T cell antigen receptors using the $V_{\beta}12$ gene segment. In addition, HIV-1 replication occurred preferentially in $V_{\beta}12$ T cells, as compared with 11 other V_{β} subsets, including $V_{\beta}8$, from normal fresh peripheral blood mononuclear cells. Both genetic background and the environment can modulate V_{β} gene expression. In children vertically infected with HIV-1, encounter of thymocytes with the viral antigen occurs early in the developmental stage, which could impact T cell receptor expression. It will be interesting to determine in children if specific V_{β} families are preferentially infected by HIV-1, and how an early viral exposure (in utero) would affect expression of V_{β} subsets.

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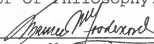
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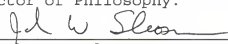
BIOGRAPHICAL SKETCH

I was born and raised in Belo Horizonte, MG, Brazil, a large city located in the mountains of my country. The first child of a family of 3, I started attending school at 3.5 years of age. I still remember that the priority of my parents was to give us the best education available. As I was growing up I attended several courses, including languages, sports, music and arts. English and swimming were the two I enjoyed the most. During high school I went to a Catholic school where mathematics was the strongest field, almost causing me to become a mathematician. Also during high school, in 1977, I spent 6 months as an exchange student in Port St. Joe, FL, where I met my husband Steve. Although inclined to follow my father's career as a lawyer, I decided to become a physician. I attended the Federal University of Minas Gerais (UFMG) where I obtained a Medical degree in July of 1985, and specialized in Anesthesiology at Hospital das Clínicas, UFMG. In 1991 I came to Gainesville, FL, and worked for a year as a visiting researcher in the Dept. of Anesthesiology at Shands Hospital. I joined the Department of Pathology and Laboratory Medicine at UF in 1992, as a Ph.D. student. At the conclusion of my degree I will return to Belo Horizonte and work in HIV-1 research, as well as with anesthesiology.

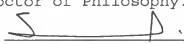
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Maureen M. Goodenow, Chair
Associate Professor of
Pathology and Laboratory
Medicine

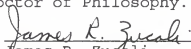
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John W. Sleasman
Associate Professor of
Pediatrics

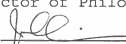
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Saeed R. Khan
Associate Professor of
Pathology and Laboratory
Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


James R. Zucali
Associate Professor of
Pathology and Laboratory
Medicine

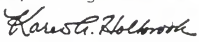
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John P. Aris
Assistant Professor of
Anatomy and Cell Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1996


Dean, College of Medicine


Dean, Graduate School